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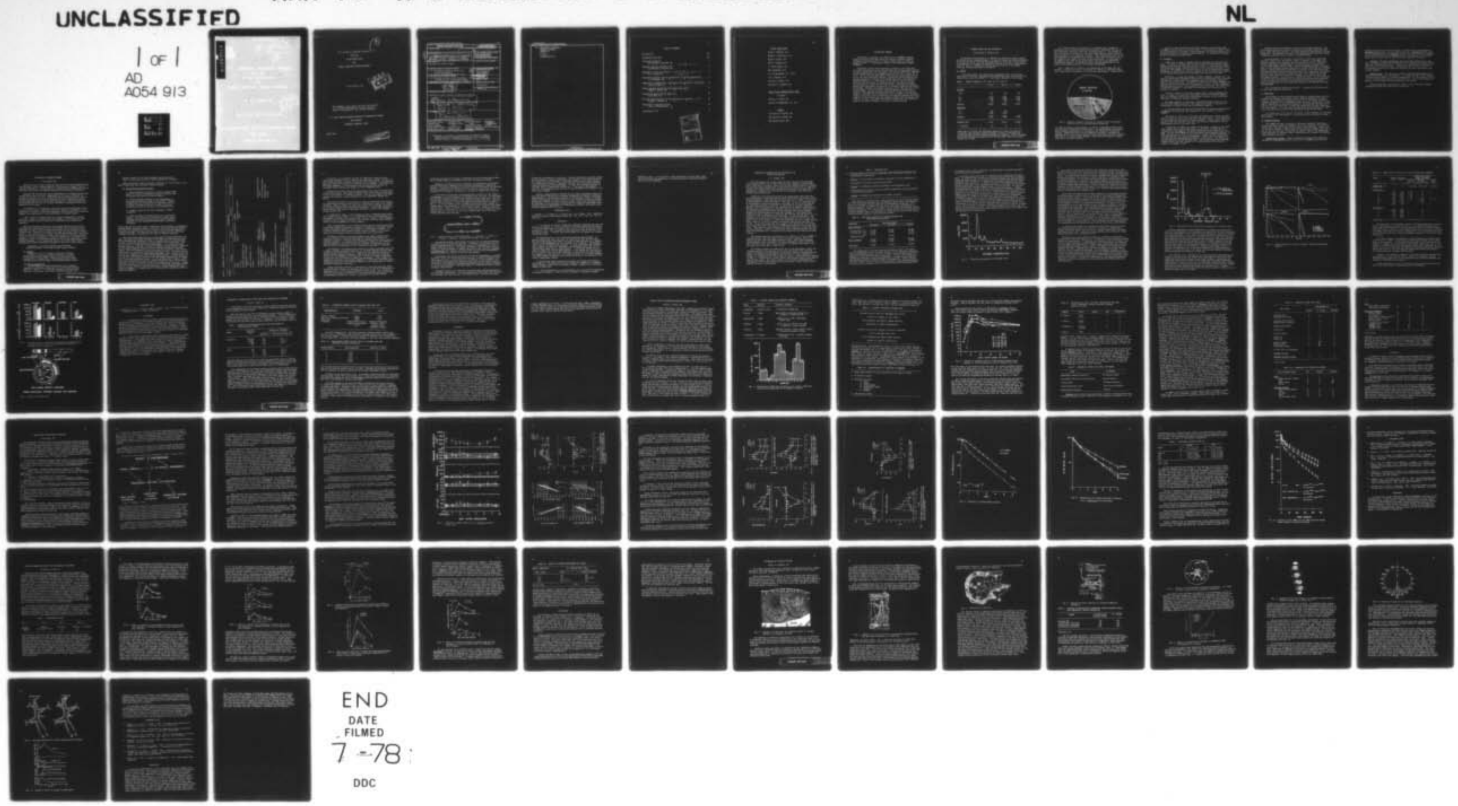

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1977 USAMRIID PLANNING SESSION

WITH THE

AD HOC STUDY GROUP

FOR

SPECIAL INFECTIOUS DISEASE PROBLEMS

27-28 October 1977

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other official documents.

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FORT DETRICK

FREDERICK, MARYLAND 21701

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Lassa fever virus			

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Progress is reported in selected areas of research in medical defense aspects of biological agents by the U. S. Army Medical Research Institute of Infectious Diseases. (cont on p. v)

19. Francisella tularensis
Coxiella burnetii
Immunology
T cells
B cells
encephalomyelitis

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INTRODUCTORY REMARKS

COL Richard F. Barquist, MC, opened the 1977 USAMRIID Planning Session with the Ad Hoc Study Group for Special Infectious Disease Problems by introducing COL P. E. Winter, Deputy Chief of Staff for Research Plans, U. S. Army Medical Research and Development Command (USAMRDC).

COL Winter relayed General Augerson's regrets at not being able to attend this meeting, but he was serving as the Acting Surgeon Gerneral and had to remain near the "hot lines." COL Winter briefed the Committee on the present status of the USAMRDC budget for the next 5 years, FY 78-FY 82. The FY 1979 and 1980 monies were recently revised upward and are now at the level previously forecast. The FY 1981 and 1982 monies were revised downward in the order of $5,000,000 each year, and represent a no-growth situation. If referenced to the value of the 1974 dollar, there has been an erosion of purchasing power. COL Winter reported that an analysis of data over the past 10 years indicated that medical research had consistently received 3% of the Army's research and development budget. However, the budget projection for FY 1981 was only 2-1/2% of this budget. He felt that the big money managers realized that a change of this magnitude would be catastrophic to some of the medical programs. They may be more sympathetic to our programs in future budget drills. This point will be tested next week when the Command presents an argument of how inflation has hurt medical programs more extensively than other R & D areas. COL Winter concluded by stating that our financial position may improve somewhat as a result of these meetings.

RESEARCH REALITIES AND PRIORITIES

COL Richard F. Barquist, MC

My objective this morning is to address the realities of money, people, facilities and the research program. The past 10 months have been an exciting period for me and as I address the above subjects I hope that I will be able to communicate to you why I am enthusiastic and why I am full of anticipation about the future of USAMRIID's research program in spite of the gloomy financial forecast presented by COL Winter.

A. Monies

In the past fiscal year which ended in September 1977, the Institute spent about $7,000,000. Approximately $8,000,000 have been allocated for the current fiscal year 1978 (Table I). This figure represents a nice change

TABLE I. USAMRIID FUNDING ($ IN 1,000) AS OF 15 JULY 77

	FY 77	FY 78	FY 79
IN-HOUSE			
ILIR	185	196	210
6.1	1,748	1,864	2,000
6.2	4,938	5,901	6,100
6.5	88	200	200
SUBTOTAL	6,959	8,161	8,510
CONTRACTS			
6.1	186	838	
6.2	1,492	1,762	
SUBTOTAL	1,678	2,600	2,486
PROGRAM TOTAL	8,637	10,761	10,996
% In-House	81	76	77

upward and if it holds, the additional monies will permit the Institute to purchase some necessary capital equipment as well as insulating the research program from the continuing erosion of inflation. Since COL Winter has already provided information for FY 1979 and 1980, there is no need to discuss this further.

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The contract monies, the funds spent to maintain the 4 biological production suites of Merrell-National Laboratories, Swiftwater, PA, and the 10-12 University contracts, have also increased (Table I). The figures at the bottom give the percentage of the total budget which is spent in-house. This percentage is very high when compared to the conceptualized ideal of the money managers in the Department of Defense. Their feeling is that a lot of the brains of the country are found in the universities. If about half of the budget is not spent at the universities, then one is not adequately tapping this resource. We defend our heavy in-house expenditures on the basis of our unique facilities for biohazard containment.

Fig. 1 shows how our resources are apportioned in big lumps over the program. A little over half of the monies are devoted to "improved prevention," which may be translated to read "vaccine development." Another 25% of

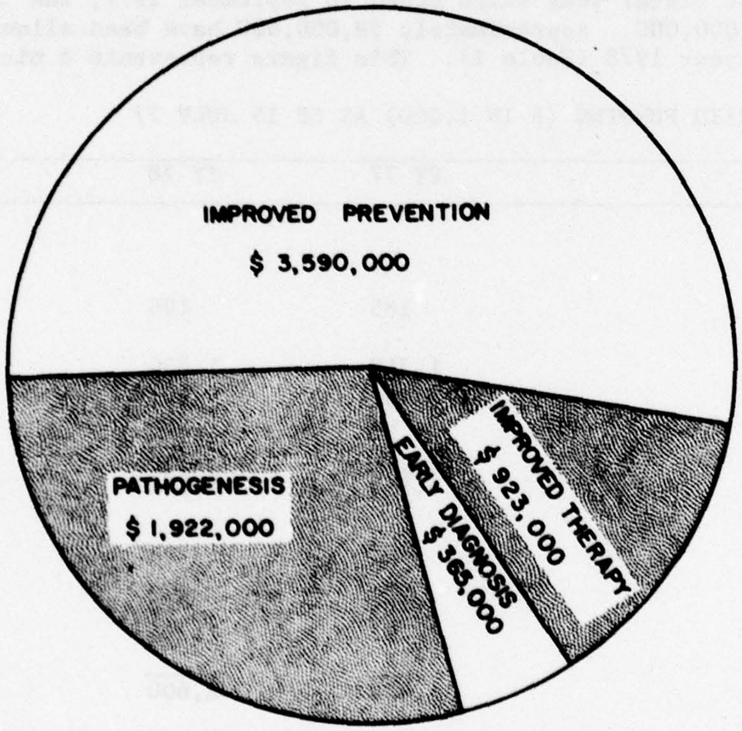

Fig. 1. Research strategy: partition of research effort by monetary costs for FY 77 to achieve mission objectives

our funds is devoted to pathogenesis, an effort to understand disease evolution and also to establish an animal model. The establishment of an animal model provides opportunities for therapeutic and preventive intervention studies. Another key area is early diagnosis; although it represents an important part of the research program, this area is small in terms of monies and people. To conclude money matters, I hope that the optimistic funding level for FY 1978 will continue.

In order to meet possible FY 1978 fund reductions, we have been asked by USAMRDC to prioritize our contracted work. In the process of doing a logical prioritization of contracts, it was necessary to do the same thing for all in-house work which the contract program supports. Dr. Beisel will say more about this during his presentation.

B. Personnel

I would like to shift to people, which is certainly our most important asset. We cannot do without money but we are nothing without people. Major changes in key personnel over the past year follow. I succeeded COL Metzger; very fortunately he has remained available to the Institute's program. He is assigned to HQ, USAMRDC, as a special project officer for BW medical defense. COL Metzger is detailed for duty here heading up our biological toxin program and is concentrating right now on botulism, a new start for this year.

COL Norton I. German was assigned as Deputy Commander, January 1977. He came from Walter Reed Army Medical Center, and is a pathologist with extensive experience in clinical laboratory management and the training of people in clinical laboratory skills. In addition to the duties of Deputy Commander, he has been specifically tasked with coordinating the effort in early diagnosis, improving quality control, the standardization of procedures, the exportation of laboratory diagnostic technology and interfacing with the users of the results of research.

LTC Peter Loizeaux is our newly assigned Chief, Animal Assessment Division. He comes to us from Walter Reed Army Institute of Research (WRAIR) and is a veterinary officer with radiobiology training; he has considerable experience in medicinal chemistry research.

MAJ James Anderson, an internist, replaced MAJ Michael Ascher in June 1977 as Chief, Medical Division. MAJ Ascher has returned to the bench to concentrate on transfer factor and cell-mediated immunity.

Dr. Richard Kenyon was appointed Acting Chief of Rickettsiology Division in September 1977 when MAJ Carl Pedersen left the Institute to join HQ, USAMRDC.

LTC Kenneth W, Hedlund, an internist and immunologist, joined our staff about one year ago and is now Chief, Bacteriology Division. He is heading up our new program on Legionnaires' disease organism. There are many other new people in the Institute and I ask that they introduce themselves to you as opportunities occur in the next 2 days.

I would like to address recruitment of personnel. Another of the realities to which the Institute must adjust is the end of the draft and the tailing-off of the number of obligated physician/officers available to the Army. In late 1976 USAMRDC was told that patient-care physician needs of the Army could not be met in 1977, and therefore no more obligated physicians could be available for research assignments. However we were promised that we could keep any unobligated physicians we could recruit. Our approach to recruiting has been along multiple lines. One obvious group were those MDs already on duty; we have retained 2 of 7 physicians who were eligible to depart in the summer of 1977.

Personal approaches by members of the staff have been productive. One civilian physician and one military physician have already joined our staff due to these individual efforts. Two others apparently have decided to accept commissions. The placement services of the Endocrine Society and the College of American Pathologists were helpful in providing leads.

An advertisement for internists interested in infectious disease was inserted 3 times in the New England Journal of Medicine in September 1977. Eighteen responses were received, and they continue to arrive. Responders include pathologists, epidemiologists, foreign graduates, academicians, interns, and husband and wife teams. PhDs have responded as well as internists. We are inviting the most promising for one-day interviews at Government expense. Medical Department counselors, 34 officers in 25 locations across the country, assist us in verifying eligibility for a commission; they answer questions about pay and rank, arrange for active duty orders, and provide personal contact with the prospective candidate at a critical time in the recruitment process. It is too soon to assess the effort. We are authorized 15 military and 3 civilian MDs for our research and patient care responsibilities. We now have 5 military physician vacancies to recruit against. I have been assured that if we can get more doctors we can probably have more authorizations.

This concludes my discussion of people. I believe you will agree with me that this represents "good news."

C. Facilities

As we prepare to open research on new agents of greater hazard, we have reviewed our biohazard containment system. National standards are being raised in the reexamination prompted by concerns for the safety of DNA recombinant research. We are upgrading filters and their surveillance, raising some P4 laboratories to P4 suited capability, and operating some areas as P4 laboratories, which were built to have this capability, but were being used for cold research. If we can gain some additional animal holding area capability, this will free at least 2 more laboratory suites for hot work.

As a reflection of our concern for safety, we have upgraded our part-time safety officer, Mr. Ralph Kuehne, to full-time safety and regulatory compliance officer.

D. Research Program

The USAMRDC has been criticized in the past for having 2 infectious disease Institutes, WRAIR and USAMRIID, which were apparently not very well coordinated. That may or may not have been a valid criticism. However, we are now in the process of doing things to give positive evidence that this is not so, that in fact the 2 Institutes are linked. Some of the significant steps undertaken to integrate these 2 laboratories in a climate of shrinking resources are as follows:

Malaria drug research. WRAIR antimalarial drug protocols requiring induction of experimental malaria will be done at USAMRIID using both our

military and civilian volunteer subjects. To date we have rejuvenated one Plasmodium vivax strain, one chloroquin-sensitive P. falciparum strain, and two chloroquin-resistant P. falciparum strains in a total of 6 human passages. As soon as the subjects have remained free of viral hepatitis for 6 months, these protocols will test the drug, WR-171-669, against the strains.

Virology. Virology programs have enjoyed strong cooperation for some time. For example, WRAIR and USAMRIID have joint programs for dengue, Chikungunya and O'Nong-nyong vaccines. A major joint virology effort has been the preparation of an arbovirus plan. Although this plan is still in draft, I have provided copies to the committee and guests for their information, and welcome comments.

Rickettsiology. The last area of finite bridge-building between Institutes is in the area of rickettsiology. Dr. Joseph Osterman, Chief, Division of Rickettsiology, WRAIR, is assuming responsibility for the scientific direction of rickettsiology research in both Institutes. We are still working out details, but the concept has been accepted and is working.

Other opportunities for building a single, strong, infectious disease program which meets Army needs will be exploited.

HIGHLIGHTS OF CURRENT RESEARCH

W. R. Beisel, MD

Again this year we hope to make the best use of the time available during this meeting to extract maximal amounts of discussion and comment from our visiting consultants. This morning will include general presentations and the afternoon will be spent in Division sessions with individual consultants.

Although this evening will not be highlighted by the Smadel Lecture given so excellently last year by Dr. Rammelkamp, we will have an informal get-together at the Officers Club. Tomorrow should be interesting also. One of the major highlights of the entire meeting will be the discussion tomorrow morning based upon the list of questions we prepared. These questions deal with problems that we feel are of major importance in planning our work and projects for the future.

As pointed out by COL Barquist, this past year has involved many transitions at the Institute. There have been major changes in personnel, in our approaches to research funding and management, and especially, there have been changes in our approaches to the prioritization of research tasks.

I will speak for a moment about this careful reexamination of annual priorities. Many of the questions we are asking you to consider are those that deal primarily with the priority of individual efforts within the Institute.

The major document dealing with our research priorities is the 1978 Scientific and Technology Objectives Guide (STOG) which is prepared by the Department of Army to outline research objectives for all aspects of Army research work. Naturally our studies fall under the medical area; from this section, I have extracted the "Concept" and "Desired Capabilities" sections as shown, limiting the statements to those which pertain to infectious disease research. In both the comments and "Desired Capabilities" paragraphs of the guidelines, there is considerable emphasis on the initial days of any future military operation. It is recognized, however, that infectious disease problems will continue throughout campaigns of more than 2 weeks duration.

Department of the Army Scientific and Technology
Objectives Guides, FY 78 Medical Combat Support Studies

(U) <u>Concept</u>
During the D to D + 15 phase of military operations in any theatre, personnel noneffectiveness due to wounds, environmental stress, combat fatigue, and some infectious diseases will be significant. BW agents may produce casualties which will severely hamper the landing/deployment phase.

(U) <u>Desired Capabilities</u>
The development of effective measures, such as medical prophylaxis and warning systems must be continued with greater emphasis. Beyond D + 15, infectious diseases, including those which are vector-borne, with longer incubation periods, will

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become a problem to the field commander, and ways must be found to reduce this problem by rapid diagnosis and treatment.

Within the medical objectives guide, priorities are listed from 1 to 16. Four of these deal with infectious disease problems.

(U) Priorities Specifically Listed:

1. Develop medical protection of soldiers against CW/BW agents to include a prophylactic system to provide long-term protection against biological agents.

3. Provide medical assistance in the development of detection and warning systems for CB warfare. Medical collaboration is also essential in research on the improvement of masks, protective clothing, collective protection for medical treatment, and warning devices.

4. Improve triage and the rapid treatment of CW/BW casualties.

6. Reduce personnel noneffectiveness due to infectious disease. New diseases not encountered in CONUS, drug-resistant variants and BW agents may produce casualties especially in the deployment/landing phase of military operations.

Notice that the very first priority emphasizes the development of prophylactic systems against biological agents. The third, fourth, and sixth priorities also deal with problems that would be faced if our troops were subjected to biological attacks. The sixth priority also includes the rare infectious diseases that might be encountered by troops in overseas locations.

Based upon these guidelines and a long series of discussions within the MRDC, the infectious disease programs at both WRAIR and this Institute have been coordinated and subjected in turn to internal Institute prioritization. In Table I are shown the research studies placed in the 3 priorities. The work in Priority I emphasizes vaccine development. It includes the basic studies necessary to achieve our goals for making vaccines against a number of new and highly dangerous viruses as well as several bacteria or bacterial toxins. Later this morning you will hear some of the details of our work in relationship to arenaviruses and Korean hemorrhagic fever. We will be initiating work in Lassa and the Congo/Crimean hemorrhagic fever viruses in the near future; Ebola (Marburg-like) virus studies should follow. Colonel Metzger is now the full time Project Officer for the R&D Command charged with the production of botulinum and anthrax toxins. He and his group will attempt to purify these toxic exoproteins in order to develop toxoids for them. We anticipate beginning work in the immediate future on the Legionnaire's bacillus. I might call your attention also to the review you have been given concerning desired work on various viruses of military importance. These documents were developed by Colonels P. K. Russell and G. A. Eddy and their respective staffs at WRAIR and USAMRIID.

TABLE I. USAMRIID GOALS: PRIORITIES

PRIORITY	VACCINE FOR: Viruses	Toxins	Bacteria	Rickettsiae
I	Lassa	Botulism (hexavalent)	Anthrax	
	Ebola	Anthrax	Legionnaire's disease agent	
	Marburg			
	Bolivian hemorrhagic fever			
	Congo/Crimean hemorrhagic fever			
	Korean hemorrhagic fever			
	Dengue I			
II	Japanese B encephalitis	Staphylococcal enterotoxins A, B, C		Rocky Mountain spotted fever
	Argentine hemorrhagic fever (Junin)	Pseudomonas exotoxins A and S		Exotic spotted fevers
	Rift Valley fever			Epidemic typhus
	Chikungunya			Q fever
	Venezuelan equine encephalitis			
	Improved therapy and early diagnosis, all agents.			
III	Western equine encephalitis		Meliodosis	
	Eastern equine encephalitis		Tularemia	
	Combined effects of radiation plus infection.			

Work in pathogenesis, immunogenesis, vaccine scale-up supporting all these goals.

In Priority II, we include vaccines for additional viruses and the rickettsial organisms listed as well as some additional toxoids. Priority II work includes studies to improve therapy and early diagnosis for all microbial agents and toxins that might be of importance in our program. COL German, our new Deputy Commander, is functioning as the Special Projects Officer to coordinate future studies with respect to early diagnostic work.

Priority III studies include the development of vaccines for several more viruses and bacteria. Basic work to support these goals, falls in this priority as do all studies of "combined injuries," such as could emerge from radiological and biological attacks delivered in some combination against our forces.

Time does not permit us to give you a complete review this year concerning the specific details of work currently in progress in each of our research divisions. However, details of these many studies can be brought out during the afternoon sessions with individual consultants. I would like, however, to mention briefly some research highlights achieved by each of the Divisions during the past year.

As indicated by Table I, the Virology Division is embarking upon studies with a number of new viruses; it is continuing to work on the development or improvement of various viral vaccines and to study a number of basic questions involved in host's immune responses at the cellular level and their possible effects on the pathogenesis of viral diseases.

Major breakthroughs in the Virology Division that you will not hear about during this morning's presentation include the adaptation of Pichinde virus in a guinea pig model leading to the production of hemorrhagic diathesis. Dr. Cole has identified some apparently stable clones of dengue I virus which appear to be attenuated, based on suckling mouse brain evaluations.

The Medical Division continues to evaluate the immune responses and protective efficacy of the many experimental vaccines being used in our Laboratory personnel. Other new vaccines are beginning their initial tests in volunteer subjects. In collaboration with WRAIR, a number of key strains of malarial parasites have been rejuvenated by passage in volunteers and will next be used in the evaluation of new antimalarial compounds in volunteers.

The program of the Rickettsiology Division is being coordinated with that of WRAIR. Further volunteer studies of our new Rocky Mountain spotted fever vaccine have now been funded by Dr. Jordan's group at National Institutes of Health and will shortly be underway. Additional questions concerning the efficacy of this USAMRIID vaccine against a variety of virulent rickettsial strains will soon be studied in guinea pigs and monkeys. Other studies are underway to accelerate the accurate diagnosis of rickettsial infections and to investigate the role of cell-mediated immunity in the pathogenesis of several different rickettsial infections.

Animal Assessment Division is continuing its work with antiviral chemotherapy and with a variety of adjuvants that may be of value for improving the immunogenicity of vaccines being developed for use in man. Dr. Liu and his group are continuing to produce data on the physiological changes which occur in experimental animals as a result of infections or bacterial toxins.

During the past year he has begun to emphasize the use of physiological methods to define supportive procedures which are of therapeutic benefit.

Similarly, in Physical Sciences Division, the biochemical and metabolic studies have begun to emphasize the therapeutic usefulness of supportive measures. The infection-induced increase in insulin secretion by the pancreas has been identified by Dr. Neufeld as a key factor in accounting for the suppression of ketogenesis in patients or laboratory animals with infection.

In an effort to determine the mechanism which accounts for the sudden disappearance of Zn from serum and its movement into the liver, LTC Sobocinski has provided conclusive evidence that during the early stages of an infection there occurs a rapid synthesis of metallothionein within the liver. This is a protein which binds zinc. A molecular model of metallothionein is shown in Fig. 1. It is a relatively short, straight-chain protein with a number of unusual amino acids appearing in fixed positions along the chain in all animal species tested. These are shown as the larger circles. These are invariant positions for a large number of cysteine, lysine and serine components of this unusual molecule. Why does the liver use valuable amino acids and energy to produce metallothionein <u>de novo</u>?

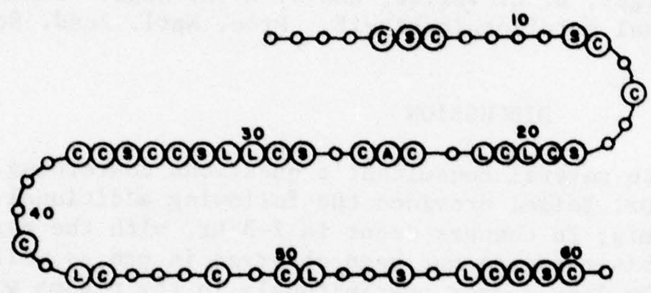

Fig. 1. Molecular model of metallothionein. C = cysteine, L = lysine, S = serine, A = arginine. [Adapted from Kojima et al. (1)].

As mentioned earlier, Bacteriology Division will be starting extensive new work with the Legionnaires bacillus. In addition, LTC Hedlund has been using isotachophoresis in a unique way to assay the appearance of soluble immune complexes in serum. Dr. McGann, MAJ Hawley, and CAPT Howell are continuing to study mechanisms of immune protection in mice exposed to <u>Francisella tularensis</u>; Dr. Canonico and CAPT Little are initiating comprehensive studies on molecular biology of activated macrophages.

Aerobiology Division has continued to study the pathogenesis of airborne bacterial, viral, and rickettsial infections and to develop therapeutic measures against them. A unique potential contribution to the studies of the Institute is the demonstration during the past year by CAPT Kastello that he can recover large numbers of pulmonary macrophages from the lungs of monkeys; he can do this repeatedly in the same monkey in a longitudinal manner without producing complications.

Pathology Division is continuing its preceptorship training program for new veterinary pathologists. As I have mentioned, studies have been initiated on botulinum toxin and soon will be on anthrax toxin. Electron microscopy

studies are continuing at a high level. Several other basic successes include the development of vascular endothelial cell cultures for the study of bacterial toxins, and the refinement of published methods using trypsin digestion of formalin-fixed tissues to rejuvenate them for use in fluoroscein-antibody tagged localizations of microorganisms. To date, this procedure has been of principal value in identifying rickettsiae. It has been of intermediate value for the arenaviruses and has yet to prove successful with respect to the arboviruses examined.

Finally, Animal Resources Division has been much strengthened during the past year and is setting up a veterinary preceptorship training in laboratory animal medicine. The division continues to provide basic support for animal surgery and handling for other Divsions; it has initiated successful breeding programs for several rare species such as moles, cotton rats and vesper mice. The remainder of this morning's presentation will emphasize hemorrhagic fever problems and recent approaches to determining the effect of vaccines on cell-mediated immunity within the host.

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DISCUSSION

1. In response to several consultant's questions concerning the type and level of Zn changes, Dr. Beisel provided the following additional information. In bacterial endotoxemia, Zn changes occur in 2-3 hr, with the maximum change occurring at 6 hr. This response has been observed in man as well as in experimental animals. Zn levels drop precipitously in the plasma with accumulation in the liver.

The administration of Zn to ameliorate infection was discussed. Dr. Beisel reported that some infections are improved and some are made worse. Much more experimental work needs to be done in this area before its administration can be recommended for the treatment of an infection. The relationship of Zn and alcoholism was discussed. In those individuals with alcoholic liver disease, Zn levels are depressed; this depression may be due to loss from the body rather than to redistribution. A consultant thought that it might be linked with alcohol dehydrogenase. It was agreed that this was one of the concepts, since Zn is most important to the function of many enzymes; for example, in acute hepatitis, the binding proteins for this metal suddenly lose their ability to hold Zn in the serum, resulting in its excretion in the urine in significant amounts.

A question was raised concerning the number of Zn atoms the molecule could take up. The answer was that the binding was composed of 3 sulfhydryl groups; moreover, there were 20 cysteine residues, 5 gm/mol with a molecular weight of about 6100.

2. The establishment of a pig endothelial cell line received considerable enthusiasm and support. Efforts are also underway to establish a primate

endothelial line. It is planned to study rickettsiae in these cells; hopefully an electron micrograph will be obtained depicting rickettsial penetration of the cell membrane.

ARENAVIRUS STRUCTURE AND THE ANTIGENICITY OF VIRION POLYPEPTIDES

J. D. Gangemi, MSC

The arenaviruses are a unique group of ether-sensitive enveloped RNA viruses whose members include the prototype virus, lymphocytic choriomeningitis (LCM) virus, as well as Lassa virus and the Tacaribe complex of viruses. Electron microscopic examinations of arenavirus-infected cells, or negatively stained virus preparations, have revealed particles which are spherical or pleomorphic, ranging in size from 90-300 nm in diameter, with 10-nm club-shaped surface projections.

Examination of ultrathin sections of all arenaviruses have shown that virus populations characteristically possess within their virions one or more 20-24-nm granules. These granules have been shown by a variety of criteria to be 80S ribosomes of host-cell origin. What role, if any, the ribosomes have in developing an infectious process is not known, nor is it certain whether all arenavirus particles, infectious or noninfectious, possess ribosomes. However, recent observations in our laboratory and in Bishop's laboratory at the University of Alabama, have indicated that the ability of arenaviruses to incorporate ribosomes may be lost during prolonged cell culture passage. Studies in various laboratories with LCM, Pichinde or Junin viruses have established that in addition to ribosomal 28S, 18S and 4-6S RNA species, 2, or perhaps 3, other viral RNA species are present in virus preparations. The sedimentation coefficients of these viral RNA species are 31S, 22S and 15S. Molar ratios of the order of 1:1 have been described for the 2 largest species, while ratios of 1:2 for the largest and smallest RNA species have been reported. It has been shown that the 2 largest viral RNA species lack methylated nucleotides; they also lack 3'-polyadenosine sequences and are not infectious. This evidence, taken together with observations of a virion RNA-directed RNA polymerase, capable of synthesizing viral complementary RNA, has led Rawls and associates (1) to postulate that arenaviruses are negative-stranded viruses.

Most arenaviruses have a predilection for rodents, it is not uncommon to find persistently infected rodent species in nature; however, some of the viruses can also cause hemorrhagic disease in man. These human pathogens include Machupo virus, the agent of Bolivian hemorrhagic fever of which we will talk more today, Junin virus, the agent of Argentine hemorrhagic fever, and Lassa virus, the agent of Lassa fever.

Our primary interest in arenaviruses was in the development of safe and efficient vaccines for human use, but we felt that before such a vaccine could be developed, more information of the basic biology of arenaviruses was needed. We therefore established 3 goals which we felt were necessary to attain as a prerequisite for future vaccine studies. These goals are presented in Table I. I would like to discuss briefly the progress which we have made in each of these areas. But before I begin, I would like to acknowledge my colleagues in the Virology Division who devoted much of their time to obtain some of the data to be presented.

TABLE I. RESEARCH GOALS

1. Characterization of structural components from both human pathogenic and nonpathogenic arenaviruses.

   Purpose: To find possible biochemical correlates to virus pathogenicity.

2. Development of an arenavirus structural model.

   Purpose: To coordinate available biochemical and biophysical data.

3. Identification of virion structural proteins responsible for the induction of protective antibodies.

   Purpose: To examine the feasibility of subunit vaccines.

With respect to the first goal, characterization of the structural components from both human pathogenic and nonpathogenic arenaviruses, we decided to make use of classical polyacrylamide gel electrophoresis techniques. In so doing, we were able to compare both the protein and lipid constituents of arenavirus members. Table II is a summary of the results obtained when highly purified preparations of 2 nonpathogens, Pichinde and Tacaribe viruses, and one pathogen, Machupo virus, were subjected to sodium dodecyl sulfate (SDS) disruption and polyacrylamide gel electrophoresis (PAGE).

TABLE II. STRUCTURAL COMPONENTS OF HUMAN PATHOGENIC AND NONPATHOGENIC ARENAVIRUSES

COMPONENTS	MOLECULAR WEIGHT		
	Pichinde	Tacaribe	Machupo
Major proteins			
Nucleoprotein (N)	68,000	68,000	68,000
Glycoprotein (G1)	65,000	38,000	47,000
(G2)	38,000		38,000
Nonglycosylated		47,000	
Minor proteins	80,000	80,000	80,000
	15,000	15,000	15,000
Glycolipid	<12,000	<12,000	<12,000

The significance of the glycosylation differences which exist among these 3 arenaviruses is not yet clear; however, it is tempting to speculate that such differences may be involved in host or target organ specificity as appears to be the case with some coronaviruses. Likewise, the presence of carbohydrate moieties in selected polypeptide chains may protect them from host-derived proteolytic enzymes during virus maturation. Future studies which will employ the use of cell mutants defective in glycosylating enzymes or drugs such as tunicamycin which can specifically block the glycosylation

of proteins will be used to determine the significance of protein glycosylation in arenavirus pathogenesis.

Our second research goal was the formation of an arenavirus structural model. To construct such a model we utilized Pichinde virus which is easy to handle and grow; we made use of several different biophysical and biochemical techniques which allowed us to examine both the internal and external organizations of virus particles. To examine internal virus components, we first treated virions with a mild detergent which gently lysed them, and then isopycnically banded the resultant substructures in a CsCl gradient. In electron micrographs numerous beaded filamentous structures were present. Similar structures have been observed by other investigators and have been reported to represent the nucleocapsid of the virus. To determine more critically if the beaded structures which we isolated from Pichinde virus did indeed represent nucleocapsid, we looked for the ability of this component to incorporate selectively lysine, which is another characteristic of nucleoproteins. Fig. 1 is a polyacrylamide gel profile of purified Pichinde virions grown in the presence of labeled lysine. As shown, the peak radio-activity indicated by N corresponds to the 68,000 dalton, nonglycosylated, structural protein of the virus. The other major structural proteins, glycoproteins G1 and G2, incorporate only trace amounts of label. These results support the concept that the beaded structures represent the major nonglycosylated protein of Pichinde virus and function as the nucleocapsid.

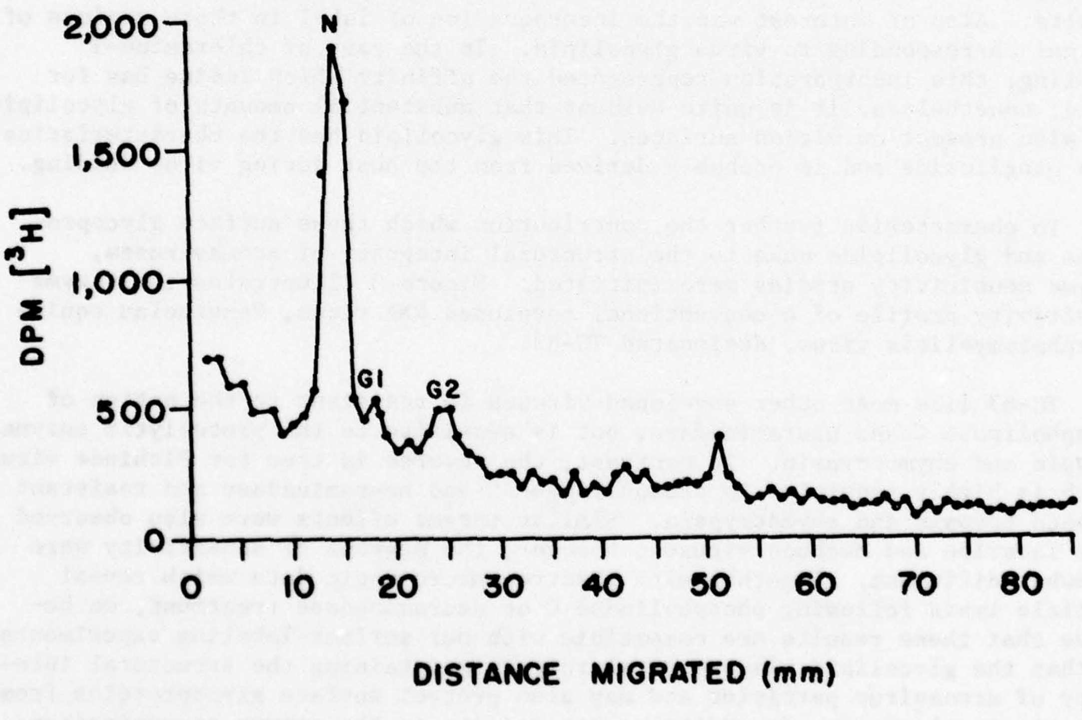

Fig. 1. [^{3}H]Lysine incorporation by Pichinde virus

In an electron micrograph obtained after detergent disruption of whole Pichinde virions, which are sometimes present inside the virus, ribosomes look identical to and band at the same density as ribosomes isolated from uninfected cells. The translational viability of these ribosomes was recently examined in studies performed at the University of West Virginia in collaboration with Dr. Herbert Thompson. It was found that when ribosomes isolated from Pichinde virions were mixed with a synthetic polyuridine template in the presence of necessary translational cofactors, peptide-bonded polyphenylalanine structures of various lengths were synthesized. Further studies in collaboration with Dr. Thompson have been designed to determine if the ribosomes which are incorporated into arenaviruses are modified during cell infection and therefore preferentially incorporated into progeny virus.

Since both nucleocapsid and ribosomal components are presumably internal virus structures, we next turned our attention to the exterior organization of Pichinde virus surfaces. To do this we had to first determine which virus structural proteins were represented on virion surfaces. This was done by labeling surface proteins on whole virions with either iodine in the presence of chloramine-T or with ^{14}C by reductive methylation. As shown in Fig. 2, glycoproteins G1 and G2 were labeled by both procedures; however, G1 always incorporated more label than G2 even though the molar quantities of G1 and G2 were shown to be equivalent. This observation did not result from a larger concentration of tyrosine residues in G1, since the reductive methylation procedure used was reactive with all free amino groups and gave similar results. Also of interest was the incorporation of label in those regions of the gel corresponding to virus glycolipid. In the case of chloramine-T labeling, this incorporation represented the affinity which iodine has for lipid; nonetheless, it is quite evident that substantial amounts of glycolipid are also present on virion surfaces. This glycolipid has the characteristics of a ganglioside and is probably derived from the host during virus budding.

To characterize further the contribution which these surface glycoproteins and glycolipids make to the structural integrity of arenaviruses, enzyme sensitivity studies were initiated. Figure 3 illustrates the enzyme sensitivity profile of a conventional enveloped RNA virus, Venezuelan equine encephalomyelitis virus, designated TC-83.

TC-83 like most other enveloped viruses is resistant to the action of phospholipase C and neuraminadase, but is sensitive to the proteolytic enzymes, trypsin and chymotrypsin. In contrast, the reverse is true for Pichinde virus which is highly sensitive to phospholipase C and neuraminadase and resistant to both trypsin and chymotrypsin. Similar enzyme effects were also observed with Tacaribe and Machupo viruses; however, the degrees of sensitivity were somewhat different. Together with electron microscopic data which reveal particle lysis following phospholipase C or neuraminadase treatment, we believe that these results are compatible with our surface-labeling experiments, in that the glycolipid plays a vital role in maintaining the structural integrity of arenavirus particles and may also protect surface glycoproteins from proteolytic cleavage. In addition, sensitivity to the enzyme neuraminadase indicates that this glycolipid may be more specifically characterized as a sialoglycolipid.

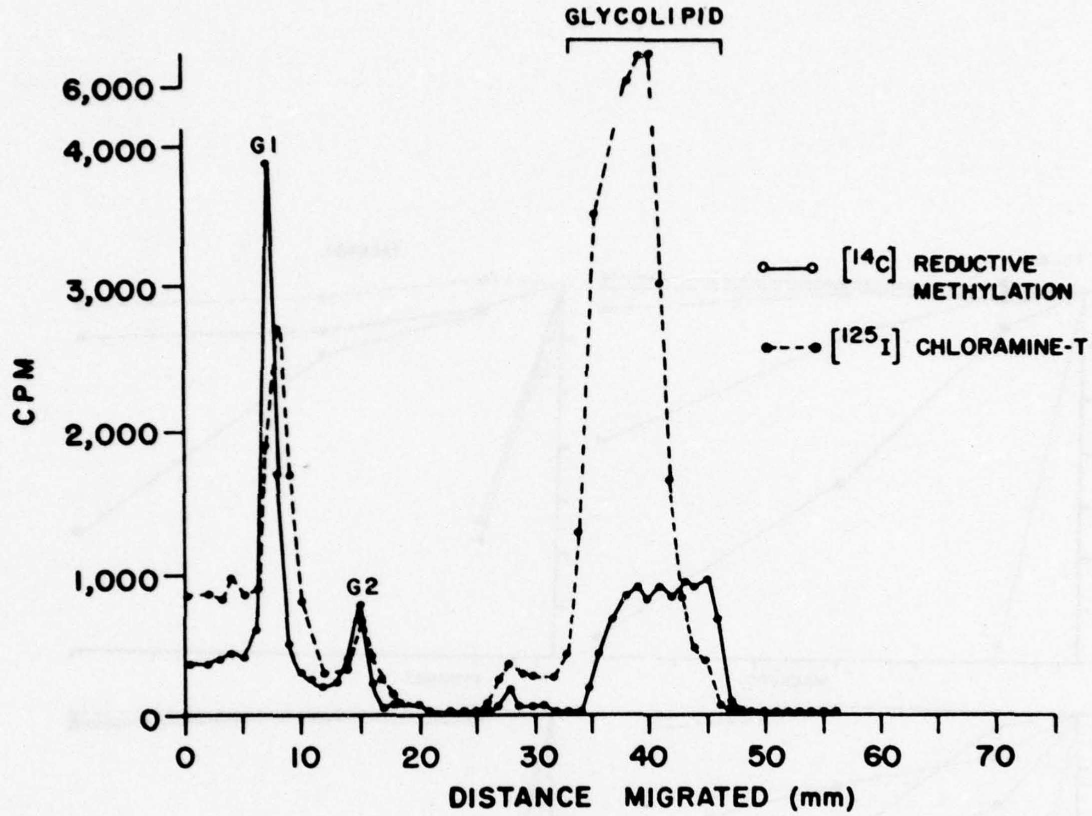

Fig. 2. Radiolabeling of virion surface components of Pichinde virus

Having superficially characterized both the internal and external proteins of one arenavirus, we next attempted to characterize which of these proteins was responsible for the induction of neutralizing or protective antibody in an infected animal. To do this, we again utilized the differential capacity which CsCl has with respect to the isolation of Pichinde virus subunits. When purified virus was gently disrupted with detergent, the resulting components were then isopycnically banded in CsCl; 3 distinct density zones containing either virus glycolipid, glycoprotein or nucleoprotein were formed. When the glycoprotein or nucleoprotein component of Pichinde virus was prepared in this manner it was possible to obtain enough subunit antigen to inoculate intradermally 150 µg of either component into guinea pigs once a week for 3 consecutive weeks. The guinea pig antibody responses following these inoculations are presented in Table III.

With the help of Dr. Jahrling and his newly developed lethal guinea pig strain of Pichinde virus, the animals were challenged one month after last inoculation and closely observed for the next few weeks. As shown on the right hand portion of this table, those guinea pigs receiving no immunogen showed signs of illness on day 10 or 11, developed viremia and died. Animals receiving only nucleoprotein showed signs of illness earlier than the unimmunized control group, developed no viremia and recovered (6/7). Animals receiving only glycoprotein subunits did not develop signs of illness, did not develop viremias, and all survived.

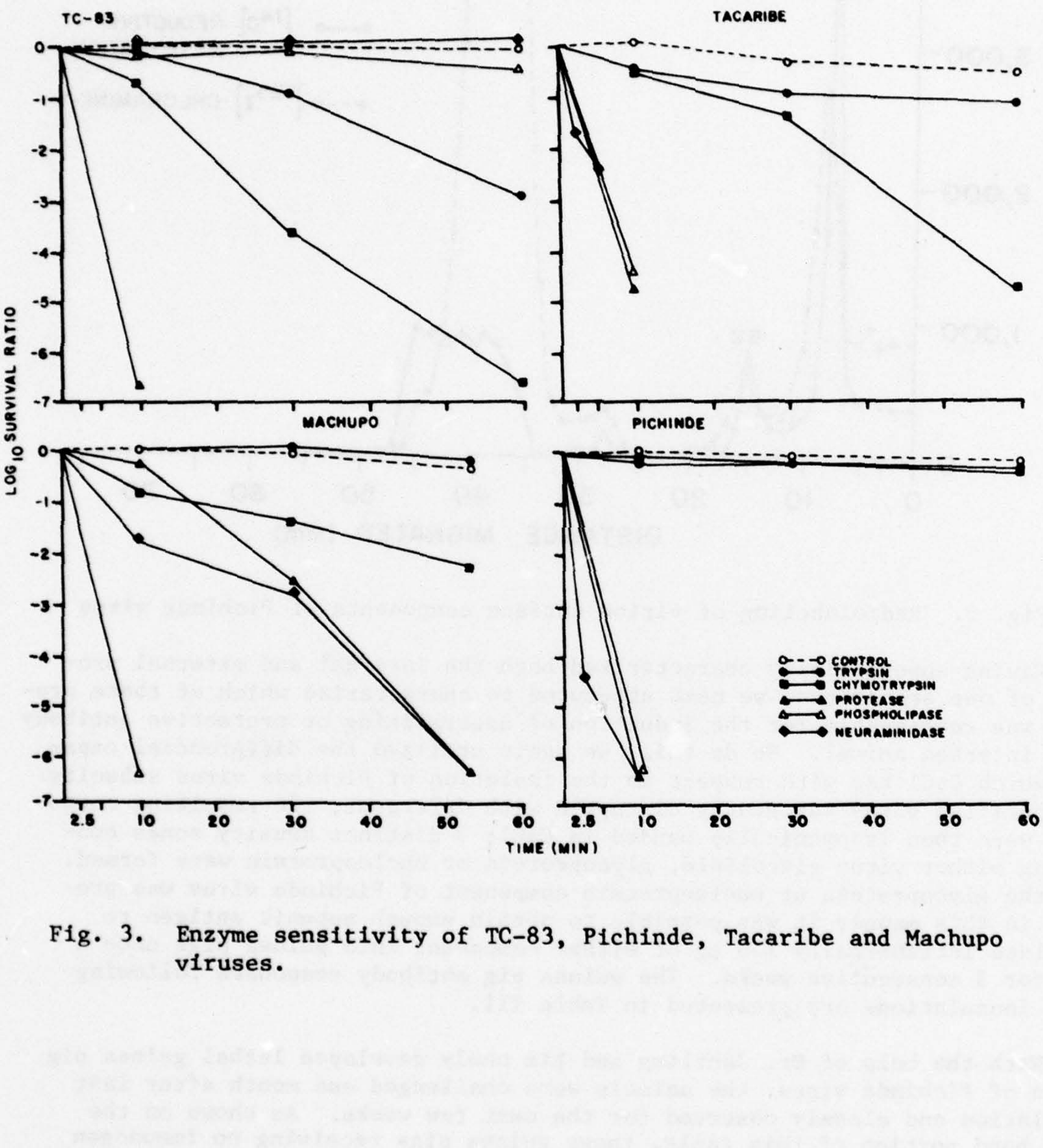

Fig. 3. Enzyme sensitivity of TC-83, Pichinde, Tacaribe and Machupo viruses.

TABLE III. IMMUNIZATION OF GUINEA PIGS WITH PICHINDE VIRUS SUBUNITS

GROUP	ANTIBODY RESPONSE		PROTECTION AGAINST LETHAL CHALLENGE		
	CF	IFA	Illness (onset day)	Viremia (log_{10}PFU/ml)	Death (days)
Unimmunized, n = 7	0	0	+ (10,11)	(3.7-5.8)	+ (11-14)
Nucleoprotein					
1	128	ND	+ (9)	0*	-
2	2048	6400	+ (9)	0	-
3	4096	12800	+ (9)	0	-
4	128	1600	+ (9)	0	-
5a	4086	1600	+ (6)	ND	+ (12)
b	ND	ND	-	(2.9)	-
6	1024	1600	-	0	-
7	1024	ND	-	0	-
Glycoprotein					
1	1024	1600	-	0	-
2	2048	3200	-	0	-
3	512	6400	-	0	-
4	256	6400	-	0	-
5	256	3200	-	0	-

* <0.7 logs

The specificity of the antibody induced by either nucleoprotein or glycoprotein immunization is presented in Fig. 4. By complement fixation (CF), assays some cross-reactivity of antibody directed against Pichinde nucleoprotein with Parana and LCM was evident, while little if any crossing of antibody to Pichinde glycoprotein occurred. Examination of this cross-reactivity with a more sensitive fluorescent antibody technique (IFA) better illustrated these differences.

The results suggest that antibody to the glycoprotein of Pichinde virus is specific and capable of protecting an animal from challenge with a lethal virus of the same type. On the other hand, antibody to the nucleoprotein of Pichinde is somewhat more cross-reactive with other arenaviruses, but is still capable of affording some protection against lethal virus infection. We are now in the process of examining the protection afforded by antibodies directed against the cross-reactive nucleoprotein antigen of Pichinde with respect to a human arenavirus pathogen, Machupo virus.

Finally, in an attempt to bring all of the structural data obtained from our laboratory, as well as from others, together in a somewhat coherent fashion, we have constructed a virus model which we believe may resemble the structure of Pichinde virus, Figure 5.

Future experiments to test the validity of this model and its relationship to the human arenavirus pathogens are now in progress.

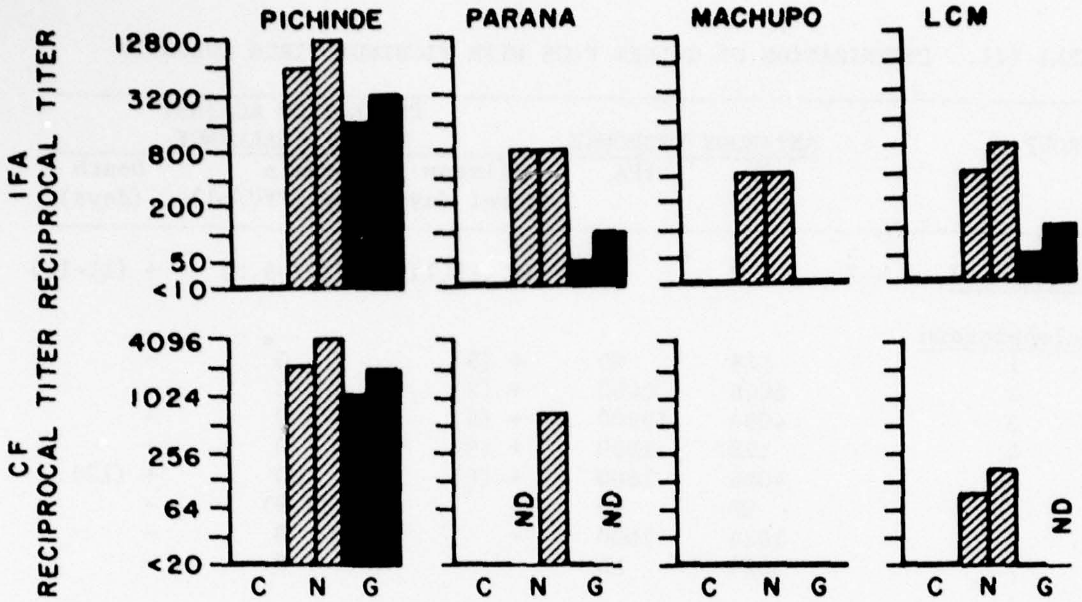

Fig. 4. Cross-reactivity of antibody from guinea pigs immunized with with nucleoprotein (N) and glycoprotein (G) subunits of Pichinde virus; controls (C)

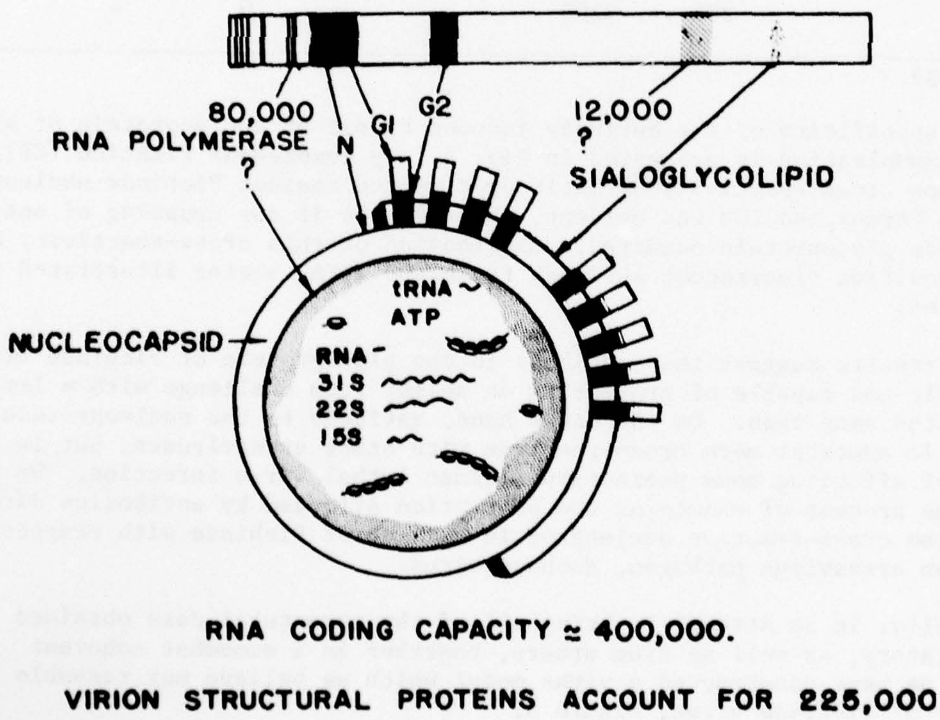

Fig. 5. Model of Pichinde virus

LITERATURE CITED

1. Ramos, B. A., R. J. Courtney, and W. E. Rawls. 1972. Structural proteins of Pichinde virus. J. Virol. 10:661-667.

DISCUSSION

1. In response to several consultants' questions concerning the source and nature of the antigen, CPT Gangemi provided the following information. The antigen used in CF tests was virus from suckling mouse brain and contained all of the antigens produced during viral replication. Efforts to perform plaque neutralization with Pichinde virus have not been successful. However, no one has successfully plaqued the virus. The point was made that perhaps purified subunits of antigen should be tested in the CF test since this might improve the specificity of the immune response.

2. A question was asked whether these observations were made on a single strain of virus. The biochemical results were obtained from the prototype Pichinde virus. CPT Gangemi would like to study the lethal model developed at this institute (Dr. Jahrling's work) to determine if similar biochemical profiles exist between nonlethal and lethal strains. It was suggested that the molecular anatomy of the virus simply reflected the history of how the virus was grown. The data generated by this extensive study do not support this concept. None of the experimental variables studied could modify the biochemical profiles. No differences were achieved except for ribosomal content, which was lost in passage in cell culture. This loss could be confirmed by electron micrographs.

ADAPTATION OF ARENAVIRUSES TO NEW HOSTS WITH INCREASES IN VIRULENCE

Gerald A. Eddy, VC

I would like to call your attention to some arenavirus data from our laboratory suggesting that this group consists of a *genetically plastic collection* of serologically related viruses.

Some time ago we found that by sequential spleen-to-spleen passage of Machupo virus in guinea pigs, the virus rapidly changed. In this instance the prototype Carvallo strain was used to assess the effect of guinea pig spleen passage upon the virulence of the virus. Following 5 such passages, a virus was derived with virulence characteristics different from the parent (Table I).

TABLE I. LETHALITY OF PROTOTYPE MACHUPO VIRUS AND GUINEA PIG SPLEEN-PASSAGED PROGENY

VIRUS	RECIPROCAL TITER	GUINEA PIG VIRULENCE	
		Dead/Total	Mean day of death ± SE
Prototype	200,000	2/6	10
	20,000	3/6	19 ± 8
	2,000	4/6	22 ± 4
	200	1/6	12
C-SP5	200	8/8	14 ± 1
	20	8/8	16 ± 1
	2	8/8	18 ± 1
	0.2	2/8	18

The data show that the derived virus C-SP5 was predictably lethal for guinea pigs and produced deaths within 2-3 weeks depending on the dose of virus used. This is in contrast to the parent virus which is inconsistently virulent for adult guinea pigs and for which dose-response curves are meaningless.

The pathogenesis of the disease produced in guinea pigs by either the prototype or C-SP5 differed also. The signs exhibited by guinea pigs inoculated with prototype virus were predominantly neurological, whereas those of C-SP5 were more typical of hemorrhagic diseases, in that thet showed earlier depression, occasional hemorrhage and death within 14-18 days without any evidence of CNS disease. Other distinguishing characteristics of C-SP5 are shown in Table II. In contrast to the prototype parent, C-SP5 was more difficult to assay by plaque formation in cell culture and was less sensitive to antiserum against prototype virus; the histopathology of the 2 diseases was also different. The C-SP5 virus produced hemorrhagic lesions very similar to those described for experimental Junin virus infections of guinea pigs.

TABLE II. DIFFERENCES BETWEEN PROTOTYPE MACHUPO VIRUS AND C-SP5

CHARACTERISTICS	PROTOTYPE	C-SP5
Plaque formation	Good	Poor
PRN titer, prototype antiserum	1:2048	1:128
$GPLD_{50}$:PFU	1:1000	1:1
Pathology	Slight liver necrosis Lymphoid infiltration in CNS	Extensive hemorrhage Necrosis: Bone marrow, liver, epithelial tissues

From this background Dr. Jahrling attempted to achieve similar results with Pichinde virus, a moderately close relative of Machupo virus and as such a member of the Tacaribe complex. He made sequential passages of Pichinde virus in guinea pig spleen (Table III). We see that with each passage of the virus the virulence increased

TABLE III. RELATIONSHIP BETWEEN PASSAGE LEVEL OF PICHINDE VIRUS AND LETHALITY FOR ADULT GUINEA PIGS

SPLEEN PASSAGE	DEAD/INOCULATED	MEAN DAY OF DEATH
0	1/20	15
1	3/10	17
2	6/10	15
3	8/10	16
4	10/10	15
8	10/10	12

and the time-to-death decreased until all guinea pigs were killed by the virus and the mean time-to-death occurred as early as 12 days. Although the pathologic observations are not complete, the guinea pigs had grossly visible hemorrhagic lesions.

The model can be very useful to us. It provides a guinea pig model of hemorrhagic disease using a virus which is relatively safe to work with and innocuous for primates. This model will permit the study of hemorrhagic arenavirus disease in a laboratory rodent that is convenient to use for such studies.

As another example of the ease with which an arenavirus can become virulent, Dr. Rosato has adapted Tacaribe virus to a high level of mouse virulence by passage in adult mouse brain. The parent virus was not particularly virulent for adult mice by intracranial (IC) inoculation. After 2-3 passages in adult mouse brain, it became highly virulent and produced severe encephalitis and death in approximately 80% of the IC-inoculated adults. Dr. Rosato's preliminary data suggest that athymic mice and x-irradiated mice are not susceptible to the virus. Thus, it appears that this system provides a useful model for immunopathologic encephalitis by a member of the Tacaribe complex.

The apparent ease with which arenaviruses can adapt to new hosts with augmented virulence must be of concern to us. This concern is heightened by the disease patterns now being seen for Argentine hemorrhagic fever (AHF) which indicate that aerosol transmission is a prominent mechanism of human infection. Recent observations in the AHF endemic area indicate that truck drivers and equipment operators have acquired infections without setting foot in the corn fields where the infected rodents live. Infections apparently occurred following inhalation of dust from the fields. We believe that aerosol susceptibility studies are important for the virulent arenaviruses in order to assess better their potential BW hazard. The results of such experiments would serve as a guide to us in vaccine development.

DISCUSSION

Mechanisms to explain increased virulence during guinea pig spleen-to-spleen passage of Pichinde virus were discussed. One point of view was that the original virus population was mixed and more virulent forms were simply selected out on passage. The general consensus was that the virus is exceedingly plastic and adapts readily to the host tissue in which it is growing. Although increased virulence was observed in the guinea pig, there was a decrease in virulence for the inbred hamster. Moreover, when the adapted virus was inoculated into 3 rhesus monkeys, they neither developed viremia nor showed the slightest evidence of disease. Similar results were achieved with Machupo virus; that is, when this strain was passaged in guinea pigs, the disease was milder in the monkey. The monkeys still became sick but symptoms were delayed and fewer animals died.

It was noted that in passing Machupo virus in guinea pigs, the shift from nonvirulence to virulence was accomplished by a change in plaque morphology, from good plaques to poor plaques. This was thought to be a rather unusual observation and questions were raised as to the plaquing substrate and whether these data could be used as a consistent virulence marker. The plaquing substrate was Vero cells. It was suggested that this observation was due to a large number of defective interfering particles in the passaged material.

A consultant noted that many scientists in contemporary microbiology are most concerned about DNA recombinant studies, the safety of these studies and the need for regulation to control and limit them. He questioned the propriety of embarking on studies to increase deliberately the virulence of nonvirulent members of a virus group known for its plasticity. For example, Pichinde virus is a Class II agent and is safe to work with; however, if it is passaged and its virulence increased, in what type of laboratory will these studies be conducted and what animal tests will be performed to maintain a profile of virulence. COL Eddy replied that the virulent Pichinde studies were performed in the hot suites. This type of experiment, perhaps, should be confined to Class III cabinets in the future. He stated that these studies were done on the assumption that the acquisition of virulence for a particular species did not alter or change the virulence for an unrelated species. This assumption is valid for the Pichinde studies. The consultant replied that serious consideration is needed if this type of study is to be extended. The work is extremely exciting scientifically, but pushes one into an unknown area with a group of microorganisms which already contain some of the most

virulent pathogens in the world. If this Institute were to make, unknowingly, a highly virulent mutant, it could make its presence known in a very devasting way. COL Eddy felt that the routine inoculation of primates during the course of such a study could determine the status of virulence and would provide the assurance that a monster was not being created.

RECENT FINDINGS CONCERNING KOREAN HEMORRHAGIC FEVER

George R. French, MSC

Korean or epidemic hemorrhagic fever (KHF or EHF)an acute febrile illness characterized by fever, prostration, vomiting, and proteinuria; it is associated with varying degrees of hemorrhage, cardiac instability and shock. The most prominent feature is obvious renal involvement. The disease first came to the attention of American physicians during the early 1950s with our involvement in the Korean war, although the same or a very similar disease had been described 35 yr earlier by the Russians in Far Eastern Soviet Russia and again 10 yr previously by the Japanese during their occupation of Manchuria. Some 8,000 cases with an average 7% fatality rate have been described in Korea, and 3,000 of these were in our own forces. Until 1970, this was a disease primarily of the military limited to localized areas north of Seoul. However, since 1970 the disease has become more widespread and moved southward; it now involves the entire Korean Peninsula. Concurrent with the southward spread has been the inevitable involvement of the civilian population. At the present time at least 800 cases of new disease occur each year, for the first time the number of reported cases in the civilian population exceeds that which occurs in the military population. Further, the disease is not limited to the Far East of Russia but occurs throughout that country, perhaps as many as 2,000 cases per year; it occurs in Finland; it has occurred at least once in Japan.

Today I would like to bring you up to date with regard to some recent advances in our knowledge of the etiology and epidemiology of this disease. The information I will present to you is primarily the result of the findings of a Korean investigator, Dr. Ho Wang Lee, a grantee from the Korea University College of Medicine.

A summary of the clinical course and laboratory findings is presented in Table I. Not shown is the prominent leukocytosis characterized by an increase in immature forms, seen early in the disease. With regard to the diuretic phase, urine output normally increases to 2 L/day, but to as much as 8 L is not unusual.

A graphic presentation of the monthly occurrence of cases seen in U.S. Forces during the years 1952-1954 is shown Figure 1. Note the bimodal peaks in late spring and fall. Although the number of cases seen in June is almost equal to that seen in November in this instance, most people would agree that the number of cases in the fall generally exceeds that seen in the spring and summer by 2-fold. Apparently the same thing holds true in Russia, especially with the Far Eastern disease.

In spite of intensive effort over the past 25 years, the etiologic agent of KHF has not been isolated. In fact, until last year there was no hard evidence that KHF was an infectious disease. We first became aware of a change change in this situation when we received the 1976 Annual Report from Dr. Lee a year ago July. In this report Lee described the detection of the KHF antigen in various tissues of a field rodent, _Apodemus agrariae coreae_ utilizing convalescent sera from KHF patients and an indirect fluorescent antibody (FA) test. The test is quite simple as shown in Table II. It can be utilized to detect KHF antibody or antigen and, as Dr. Lee has subsequently shown, is

TABLE I. CLINICAL COURSE AND LABORATORY FINDINGS

PHASE	DURATION	PRINCIPAL FINDINGS
Incubation	15 days (7-46)	↑ Irritability toward end
Febrile	5 days	Hyperthermia, petechiae, severe proteinuria, ↑ serum LDH (2X)
Hypotensive	3 days	↑ Hematocrit (to 70%), hemorrhage, shock (25%)
Oliguric	4 days	↓ Urine output (< 500 ml), ↑ K, BUN + creatinine, acute azotemia
Diuretic	5 days	Symptoms resolve, ↑ urine output, fluid + electrolyte balance critical
Convalescent	4-6 weeks	↓ Sedimentation rate, ↑ specific gravity of urine

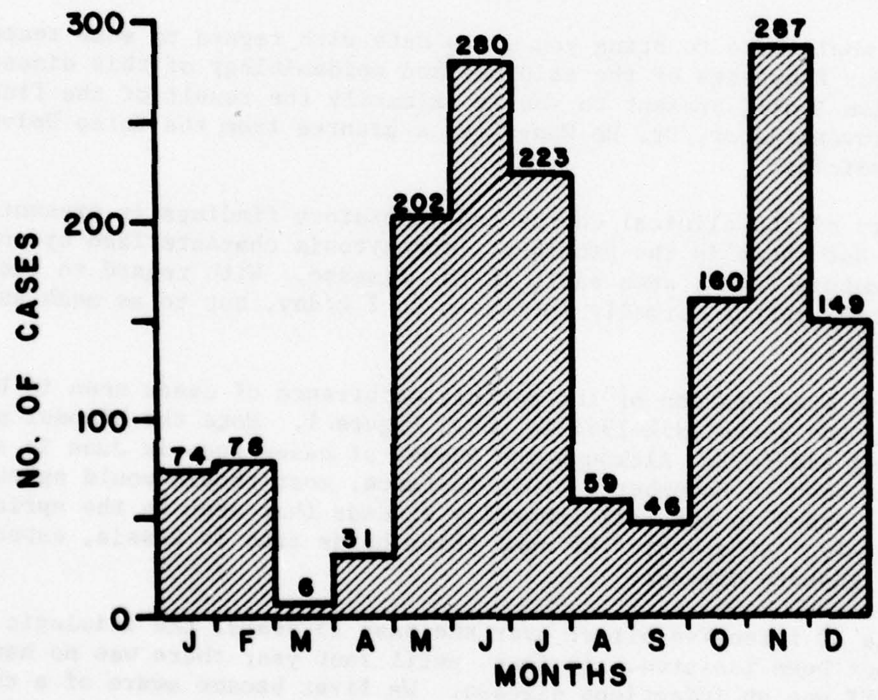

Fig. 1. Distribution of KHF cases by month in U.S. Forces, 1952-1954
(From US Army Hemorrhagic Fever Commission Reports)

convalescent sera from KHF patients and an indirect fluorescent antibody (FA) test. The test is quite simple as shown in Table II. It can be utilized to detect KHF antibody or antigen and, as Dr. Lee has subsequently shown, is

TABLE II. INDIRECT FLUORESCENT ANTIBODY TEST

Antibody acetone-fixed FA + Apodemus lung section
+
Dilution of unknown or test serum
+
FITC-conjugated anti-species immune globulin
↓
Extinction of specific fluorescence

Antigen acetone-fixed unknown tissue/cell suspension
+
8 units human anti-KHF serum
+
FITC-conjugated anti-human immune globulin
↓
Presence of specific fluorescence

disease-specific. The procedure is tedious, in that tissue (normally lung) sections are required, and the rodent must be trapped in the wild. Efforts to colonize this animal have so far been unsuccessful. The agent as detected in Apodemus is infectious and can be transmitted to clean Apodemus in the laboratory. This, of course, requires a clean source of Apodemus. Dr. Lee has solved this problem by demonstrating that Apodemus captured on offshore islands are uniformly free of the disease. Infection of these rodents is established in the laboratory by intramuscular, subcutaneous or intralung inoculation of FA-positive tissue suspensions obtained from rodents trapped in the endemic area. Characteristics of laboratory infection of Apodemus are shown in Table III. Note that peak fluorescence requires 20 days, that infection does not result in

TABLE III. CHARACTERISTICS OF INFECTION IN APODEMUS

1. Lung tissue positive 11 days postinfection and peaks at 20 days
2. No apparent disease
3. FA-positive tissues
   a. Lung
   b. Kidney
   c. Parotid gland
   d. Submaxillary gland
   e. Intestine
   f. Bladder
4. 33% develop antibody

detectable illness and that less than 1/3 of the infected animals ever develop antibody. Lung is uniformly the best tissue to demonstrate optimal fluorescence.

Having established the presence of KHF antigen in Apodemus tissues, Lee's next efforts were directed toward studying the human immune response. There are several things of interest I wish to point out in Figure 2.

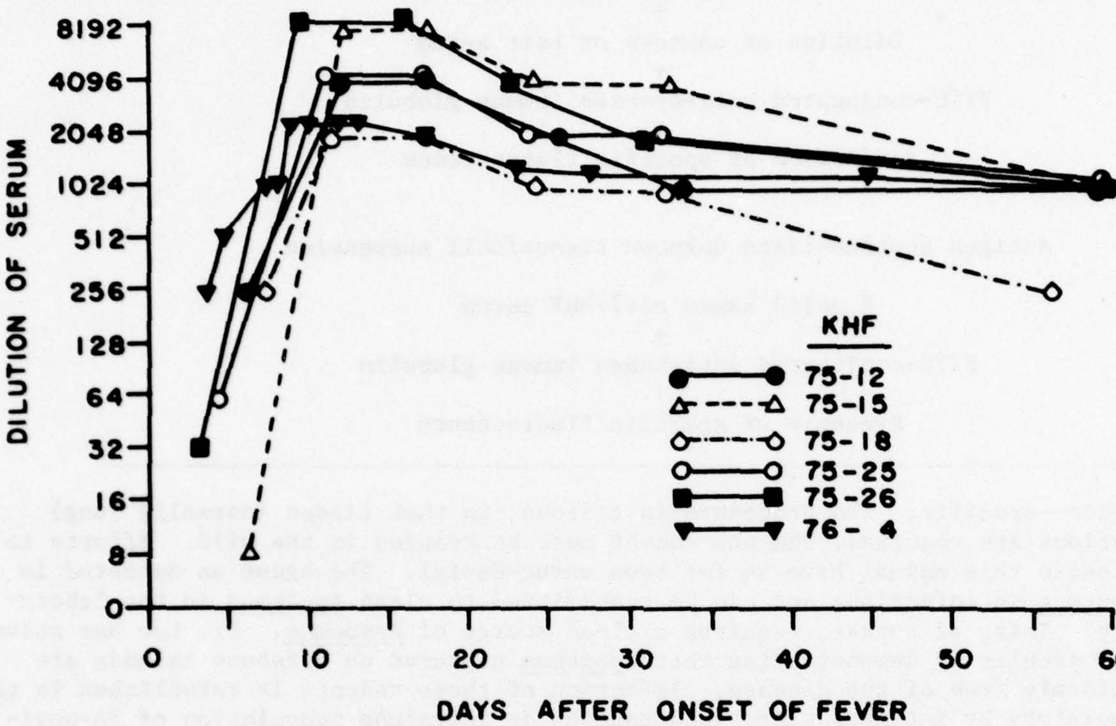

Fig. 2. Indirect FA antibody titers of 6 KHF patients against Korea antigen no. 75-191 during the course of illness (after Lee)

Note the height of the titers and the fact that all 6 have detectable antibody on the first day after onset of fever. In fact, all but a very few of several hundred patients examined to date present this way. Dr. Lee has had difficulty isolating the agent from human specimens; this is perhaps a possible explanation. Titers persist at slightly reduced levels for at least 5 yr; Dr. Lee has had no difficulty demonstrating antibody in patients sera at 15 years.

Dr. Lee had established to his own satisfaction that the test was specific for KHF by examining large numbers of sera from typical KHF patients within Korea, which were always positive, and contrasting them to various other non-KHF patients, some with other kidney diseases, which were always negative. His next task was to convince the rest of us. This he did by soliciting coded sera from various people outside Korea. Of very great interest to us was the fact that he was able to get sera from diseases thought to be the same as KHF but originating in other countries. The sera tested and where they originated are shown in Table IV. I should point out here that the 7 positive tests for sera

TABLE IV. RECOGNITION OF KHF FA IN CODED CONVALESCENT SERA FROM VARIOUS HEMORRHAGIC FEVERS WITH RENAL SYNDROME (After H. W. Lee)

SOURCE	ORIGIN	NORMAL	HFRS	FA-POSITIVE
K. Johnson	Korea		7	7
	U.S.A.	7		0
J. Casals	Russia		4	4
	U.S.A.	4		0
J. Lahdevirta	Finland		10	9
	Finland	5		0
T. Okuno	Japan	–	–	+

submitted by Dr. Johnson are, I believe, aliquots of the same serum from one patient. I do not know how many serawere submitted by Dr. Okuno, but these sera are important in that they originated from a "one-time" occurrence of the disease in Japan near Osaka, Honshu Island, about 10-15 yr ago. Thus, we can say with certainty that the diseases, which all present similarly to KHF, are also serologically related to KHF (Table V).

In summarizing the findings utilizing the indirect FA test we can now say that A. agrariae coreae is one animal reservoir of disease, and further, FA-positive Apodemus tissue is infectious. Other species or subspecies of the genus native to nonendemic areas in Korea are susceptible to infection. With regard to the human infection, we know that the humoral immune response is "normal" and that subclinical infection is a rare event. Acute-phase blood from KHF patients has yielded an infectious agent. Finally, the etiologic agent of KHF is serologically similar, if not identical, to the agents of other hemorrhagic fevers with renal syndrome.

TABLE V. HEMORRHAGIC FEVERS WITH RENAL INVOLVEMENT

NAME	OCCURRENCE
Hemorrhagic nephroso-nephritis	Far Eastern Russia
Hemorrhagic fever with renal syndrome	European Russia
Song-go fever	Northeast Manchuria
Korean hemorrhagic fever	South Korean peninsula
Nephropathia epidemica	Finland

Apodemus species have always been suspect reservoir animals because their population dynamics closely correspond to the appearance of disease, i.e., the

wild population peaks in the early summer and again in the fall. Dr. Lee has shown that the frequency of positive lungs in Apodemus trapped in hot spots of disease peaks in May and again in October slightly before the peaks of human disease. Other rodents native to endemic areas in Korea that have been tested, but so far found negative, include the house mouse, ground squirrels, a Microtus species, the Korean hamster, moles and the shrew.

In October 1976 I visited Dr. Lee at his laboratory in Korea. The purpose of my trip was 2-fold: to learn more about his findings utilizing the techniques and to bring infectious material back to USAMRIID so that we might establish the technique here and apply our greater resources in those areas Dr. Lee was not prepared to or could not deal with. Dr. Lee returned my visit in January and brought even more material with him. He had an assay system but did not have a model of disease. We decided to look at this aspect first and utilized some of those resources I mentioned earlier, subhuman primates. In cooperation with MAJ Stephen, we found that KHF would indeed infect several species of monkeys, in fact, all species that we tried; however, these animals underwent self-limiting infections without apparent disease. This finding in itself was exciting even though we still did not have an animal model, and further, it gave us an opportunity to come up to "snuff" with the indirect FA technique. This we did; now some 10 months later, we feel quite comfortable with the test. We now faced our assay problem here at USAMRIID. Apodemus had not and still has not been colonized. Monkeys could, if necessary, be utilized but this of course was too expensive and impractical. We therefore decided to take another look at laboratory rodents and cell cultures. The next series of tables show the combined results of several laboratories' contributions. None of the various species of laboratory animals tested (Table VI) became ill when inoculated with infectious material but several did produce antibody. The positive antibody responses for guinea pigs, rabbits and white rats were obtained by Dr. Lee while those for the 3 species of monkeys were obtained here at USAMRIID. The monkey experiments incidentally, were very exciting, in spite of the fact that no disease developed; this was the first evidence that any animal other than man or the reservoir host Apodemus could be infected. The next positive finding at USAMRIID was in the South American rodent Calomys callosus. The lungs of these animals becamse FA-positive 18-20 days postinoculation with infectious material from Apodemus. It is interesting that in every aspect so far examined, infection of Calomys mimics that of Apodemus. More importantly, for the present at least, it gives us an assay system. At the same time as the animal experiments were in progress, we were also examining a large variety of cell cultures for cytopathic effect (CPE), plaque formation and FA antigen. Primate and nonprimate preparations and cell lines tested are shown Table VII. Dr. Lee has had inconsistent results with Apodemus embryo cells which sometimes develop fluorescent antigen about 14 days postinfection, and we have had some success with Calomys kidney cells. The major stumbling block appears to be one of selecting the appropriate epithelial-like cell and preventing overgrowth by the spindle-shaped fibroblast-like cells. This effort is, of course, being actively pursued in an ever expanding list of cell lines.

In summary, we believe the etiologic agent of KHF is probably a conventional virus. We are optimistic enough to think that we are only a few months away from being able to adequately describe this agent and to having

TABLE VI. LABORATORY ANIMAL HOST RANGE

TEST ANIMAL	DEVELOPMENT OF		
	Disease	FA Antigen	Antibody
Suckling mouse	0	0	0
Weanling adult mouse	0	0	0
Suckling syrian hamster	0	0	0
Weanling syrian hamster	0	0	0
Weanling MHA hamster	0	0	0
Guinea pig	0	0	+
NZ white rabbit	0	0	+
Cotton rat	0	0	0
White rat	0	0	+
Squirrel monkey	0	NT[a]	+
Rhesus monkey	0	NT	+
Cynomologus monkey	0	NT	+
N. American vole (<u>Microtus</u>)			
<u>Calomys</u> <u>callosus</u>	0	+	±
<u>Apodemus</u> <u>agrariae</u> <u>coreae</u>	0	+	±

[a] Not tested

TABLE VII. LABORATORY CELL CULTURE HOST RANGE

CELL CULTURES TESTED	CPE	PLAQUES	ANTIGEN
<u>Human</u>			
Human embryonic tissue	0	0	0
HELA	0	0	0
Human amnion	0	0	NT[a]
WI-38	0	0	0
<u>Subhuman primate</u>			
Rhesus monkey kidney	0	0	0
Vero	0	0	0
LLC-MK2	0	0	0
BS-C-1	0	0	0
Rhesus monkey lung	0	0	0

Avian			
Chick embryo fibroblasts	0	0	0
Duck embryo fibroblasts	0	0	0
Nonprimate mammalian			
Porcine kidney	0	0	0
Porcine aorta	0	0	0
Baby hamster kidney (Syrian)	0	0	0
Chinese hamster kidney	0	0	0
MD dog kidney	0	0	0
N. American vole embryo	0	0	0
Apodemus embryo	0	0	±
Calomys lung	0	NT	0
Calomys kidney	0	NT	+

[a] Not tested

available an *in vitro* assay system. We do not have an animal model of disease, although we have not exhausted the possibilities; it does appear that this is going to be difficult. Many questions still remain about the epidemiology of this disease and the mechanics of how it is transmitted to man. Although *Apodemus* is most probably one animal reservoir, we have reason to believe it is not the only one. These and other remaining questions are being actively pursued here and in Korea.

DISCUSSION

A consultant suggested that *C. callosus* urinary bladder cells be used for growing the agent, since they could be maintained more readily in the presence of fibroblast contamination than renal tubular cells of endothelial origin. The suggestion was enthusiastically endorsed by LTC French.

Since KHF is more widespread than formerly considered, and has been identified as extending across the northern tiers of Asia into Europe, the possibility of it being present in North America was discussed. One of the consultants pointed out that this was highly unlikely, since members of the genus *Apodemus* do not exist here.

The appearance of the fluorescent antibody was described as fine, dustlike, diffuse particles which do not project well; therefore, no pictures were provided. In order to obtain the reaction, the test must be performed early in the infection.

A consultant described a case history, which must be considered anecdotal because of insufficient data; there is a patient in Indiana suffering from severe, chronic renal insufficiency, who is a candidate for a kidney transplant. The patient gives a history of having had KHF while serving in Korea in 1950. There is nothing else in the history to account for the present difficulty. The Institute tested his serum in 1976; he does have antibodies. The important point is that, although KHF is not considered to cause renal failure at a later date, this case is rather suggestive of a relationship.

COAGULATION STUDIES DURING INFECTION

David Wing, CPT

The coagulation system of blood is an integrated multi-enzyme biochemical pathway responsible for maintaining the physical integrity of the circulation, protecting it from damage which would interrupt its vital transportation function throughout the body. Studies of coagulation system disfunctions due to, or associated with, infectious disease, center around the syndrome of disseminated intravascular coagulation (DIC). DIC is mediated by apparent simultaneous activation of the coagulation, fibrinolytic and kinin generation systems. The syndrome has been described as a complication of an increasing variety of systemic diseases, including infections. A listing follows:

1. Complication of infectious disease caused by every class of etologic agent.
   a. Bacterial: the most prevalent cause of DIC is in association with infections by gram-negative rods, meningococcemia and Klebsielia pneumoniae; DIC DIC is known also to occur secondary to infections with gram-positive organisms.
   b. Rickettsial: primarily Rocky Mountain spotted fever (RMSF).
   c. Viral: DIC has been reported in the literature as a complication of numerous viral infections: herpes, rubella, smallpox, and various viral hemorrhagic fevers.
   d. Mycotic: histoplasmosis and aspergillosis.
   e. Protozoal: especially malaria caused by Plasmodium falciparum.
2. Neoplasm: prostate and leukemia, both due to the disease itself and with secondary aspergillosis.
3. DIC was first clinically recognized resulting from obstetric complications such as premature separation of the placenta, and the retained dead fetus syndrome.
4. DIC is often described as accompanying vascular disorders, that is any of the shock syndromes, and stasis resulting from intravascular accidents.
5. Massive tissue injury or necrosis, whether primary, or secondary to another disease process can lead to DIC. For example, DIC is often described secondary to severe cirrhosis, and may arise from injury to the kidneys, adrenals, lungs or pancreas.
6. Miscellaneous

Over the last few years, several investigators have worked in a loose cooperative group to investigate changes in the coagulation, and related systems, arising subsequent to infectious diseases of interest at USAMRIID, to develop animal models for the systematic study of DIC, and to explore treatment regimens alternative to those in current use.

Several diseases have been studied in this regard, measuring changes in coagulation function arising during the course of infection, beginning with a collaborative study with people from WRAMC on the pathophysiology of yellow fever in the early 70s. More recent work has included investigations of coagulation changes associated with sepsis in rhesus monkeys infected with Streptococcus pneumoniae and RMSF. Guinea pig and monkey animal models were examined for RMSF as well as cases of laboratory-acquired infection among laboratory personnel in this Institute.

Preliminary studies were conducted on Bolivian hemorrhagic fever in monkeys. These were extended in my own work with gram-negative sepsis in rhesus monkeys infected with Salmonella typhimurium, which was used as a model for the DIC syndrome. We investigated the pathophysiology of the syndrome using protein turnover studies of selected coagulation-related proteins. We also attempted to study a new type of treatment of DIC using aprotinin instead of heparin.

I would like to review the progress made in these recent studies toward understanding the reasons behind coagulation changes accompanying infections. Before reviewing specifics, let us refresh our memories about what evidence is used to suggest the presence of DIC.

The current wisdom concerning the pathogenesis of DIC is shown in Fig. 1.

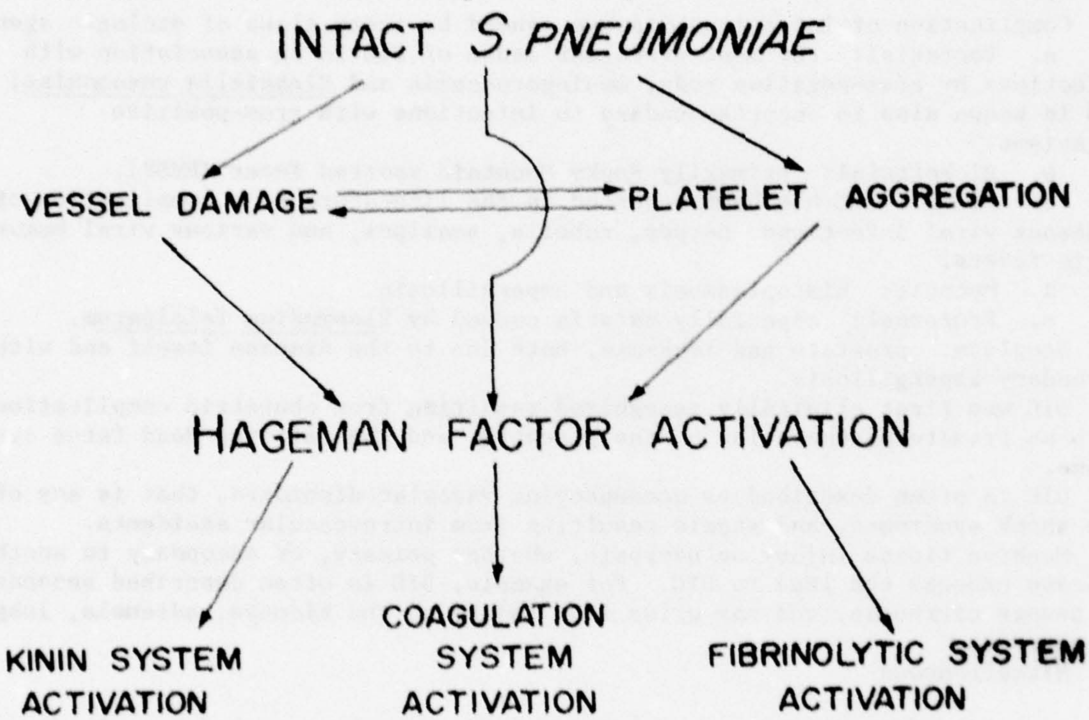

Fig. 1. Pathways of DIC

It is based on clinical experience, and the results of in vitro studies on the pathways of the systems shown, coagulation, fibrinolytic, and kinin. Injury of endothelial tissue, platelets or red cells is thought to lead to activation of the coagulation system. Coagulation factor 12, Hageman factor (HF), plays a central role in activating the kinin and fibrinolytic systems. Activated HF activates prekallikrein, the zymogen of kallikrein, an enzyme which liberates the vasoactive peptide bradykinin from high molecular weight kininogen. Activated HF also mediates activation of plasminogen to plasmin.

Activation of these systems is suggested by various laboratory tests. Activation of the coagulation system is implied by an increased prothrombin time (PT) or partial thromboplastin time (PTT), decreased platelets, or decreased fibrinogen. Activation of the fibrinolytic system is indicated by

the presence of fibrin degradation products (FDP) in the serum. Activation of the kallikrein-kinin system is conveniently measured through decreased PK levels. The pattern of laboratory tests demonstrating simultaneous activation of these systems is considered diagnostic for DIC, and is often the basis upon which treatment is ordered. We look for this same pattern of laboratory tests in data from animal models of disease, to identify the presence of a process similar to DIC observed in humans.

Let us begin our review of recent coagulation work at the Institute by discussing the studies of viral diseases. In the animal disease model studies of viral hemorrhagic fevers described by COL Eddy, pathologic examination of monkeys and guinea pigs at necropsy has provided evidence consistent with DIC, at least in the later stages of acute infections. Coagulation studies in these animals during the infection have proved quite difficult logistically. The necessity for high containment of these viruses and the infected animals, severely limits what can be accomplished efficiently. A preliminary study of coagulation changes in rhesus monkeys infected with Machupo virus was done in 1976 by CPT Steven Scott. He found decreased platelets, moderate amounts of FDP, and essentially normal fibrinogen levels during the early and middle phases of the infection. Interestingly, PTT were very much prolonged from the beginning of the illness, while the PT of the infected monkeys remained normal until just before death. DIC was thought in this case not to contribute greatly to the disease until the terminal phase.

Pneumococcal sepsis was studied by Hawley et al. (1), and in normal and asplenic rhesus monkeys infected with S. pneumoniae. They found clinical evidence of DIC in only a few of the infected monkeys, but laboratory changes included depressed platelet counts, lowered PK levels, and increased FDP, which correlated with the peak in bacteremia. The capsular polysaccharide of the organism could be measured in the serum, but peaked 1-2 days after the peak bacteremia. No significant depression in complement factors was observed by immunodiffusion.

They concluded that the capsular polysaccharide itself, immune complexes, and complement were not primary factors in initiation of DIC in association with this infection. Intact pneumococci may be catalysts for the development of DIC. Most importantly, they obtained evidence of activation of HF-dependent systems in all the infected monkeys, whether or not petechial rash, the clinical sign of DIC, was observed.

Over the past several years, RMSF has been studied extensively. Animal models for this disease have been developed in monkeys and guinea pigs. There has also been some experience with several cases of laboratory acquired infections among personnel, the report of which is being published.

Harrell in 1949 (2) distinguished between two classes of RMSF infections in humans. The first was an acute fulminating infection, in which death was thought to be due primarily to toxic effects of the rickettsiae themselves. The second, was a more prolonged infection, where antigen-antibody complexes and complement played an important part in the pathophysiology of the disease. Moe et al. (3) found two phases of the disease in rhesus monkeys infected with Rickettsia rickettsii, as in humans. They observed mild thrombocytopenia,

increased FDP, and increased fibrinogen levels, which coincided with peak rickettsemia in acute, fatal infections. In mild, nonfatal infections, activation of complement later in the course of the infection appeared to contribute to the severe arteritis which developed.

In the guinea pig model of the disease, these same investigators (4) found that coagulation changes were most apparent early in the course of the infection, coincident with the increasing rickettsemia, which peaked on day 5 of the infection. In Fig. 2 the pattern can be seen of depressed platelets, increased FDP, and prolonged clotting times indicative of activation of the coagulation and fibrinolytic systems, which are consistent with DIC.

In following the 5 cases of laboratory-acquired RMSF among laboratory workers here, Yamada et al. (5) had the opportunity to measure specific kallikrein-kinin system activation in patients. None of the patients had severe illness; there were minimal coagulation changes. However, evidence for specific kinin system activation was found. Fig. 3 shows 4 measures of kinin system activation. Using the method of Colman et al. (6) based on hydrolysis of tosylarginine methyl ester, we measured free kallikrein and prekallikrein levels during acute illness, and at late convalescence. The shaded areas represent 2 SD about the mean of 10 noninfected controls. Free, spontaneous kallikrein activity was increased, and prekallikrein was depressed during acute illness.

Plasma kallikrein inhibitor was depressed during acute illness, probably due to consumption from increased PK activation; the kininogen level, expressed as potential kinin-releasing activity, was also depressed during illness.

My own work on Salmonella has focused on development of an animal model for DIC associated with infection, which would permit a systematic study of the pathogenesis of the syndrome.

Based on an earlier report by Kastello and Spertzel (7), Dr. Beisel suggested that sepsis in monkeys infected with S. typhimurium might be an appropriate vehicle to induce DIC. Indeed, this model of gram-negative sepsis has proved quite useful in our studies. We have been able to measure the metabolic behavior of selected proteins from the coagulation-related systems during sepsis-associated DIC, using the technique of protein turnover studies.

In our model, 4-6-kg rhesus monkeys are infected with about 10^9 S. typhimurium by IV injection, while noninfected control animals are given saline. Approximately 50% of infected monkeys develop petechial rash; this we use as a clinical sign of DIC. The rash first appears 1-2 days following infection and lasts an average of 4-5 days. This model is especially useful in that under these circumstances, the infection is accompanied by very low mortality, even among monkeys with severe rash. Of approximately 50 infected monkeys only one has died of infection associated with DIC. The monkeys spontaneously recover in 8-10 days even without antibiotic treatment. The next several figures summarize the laboratory findings in these monkeys.

Infected monkeys develop fever and significant, although transitory, leukopenia, despite the apparent significance of the changes in the controls (Fig. 4).

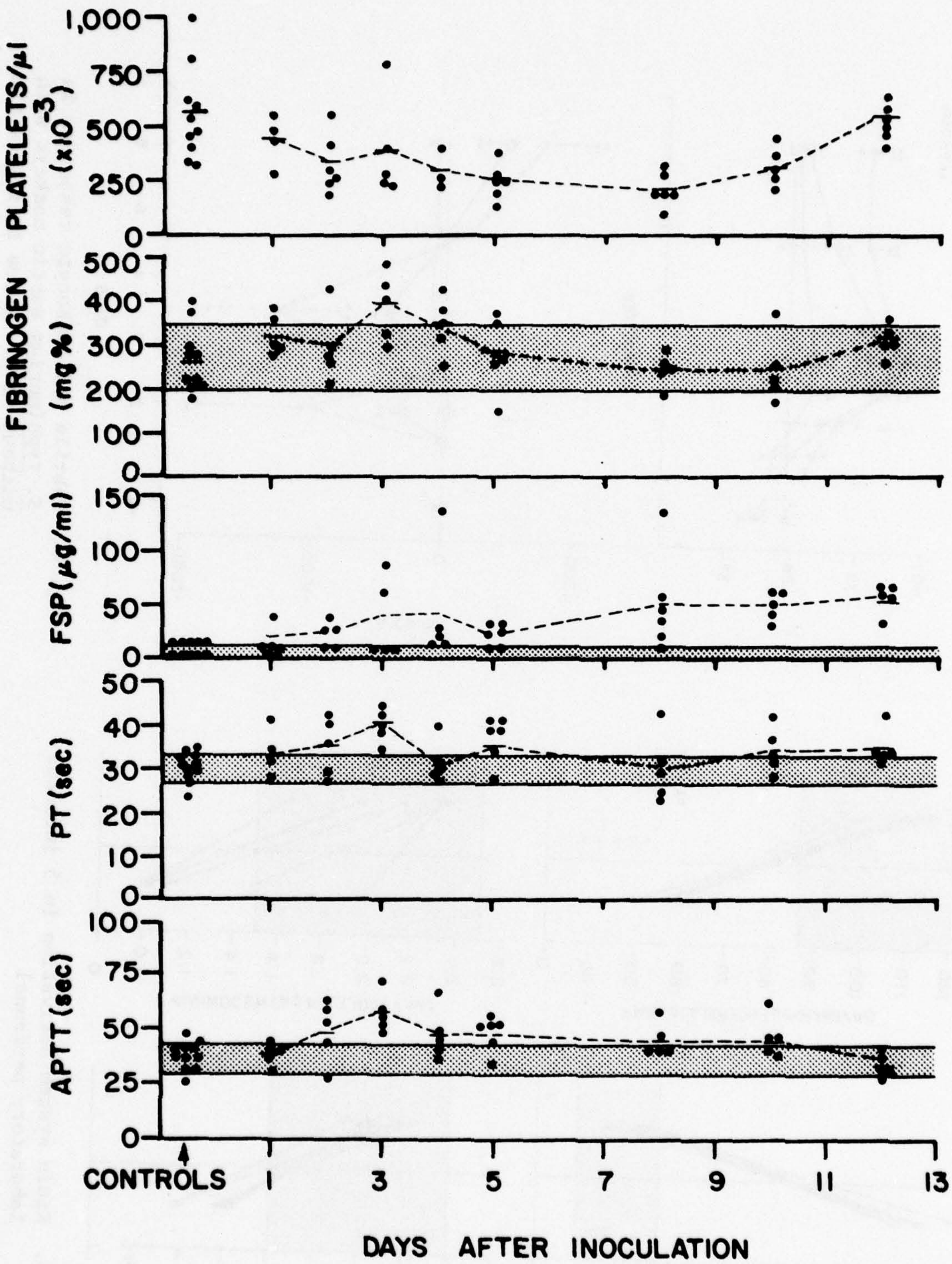

Fig. 2. Laboratory changes observed in R. rickettsii-infected guinea pigs

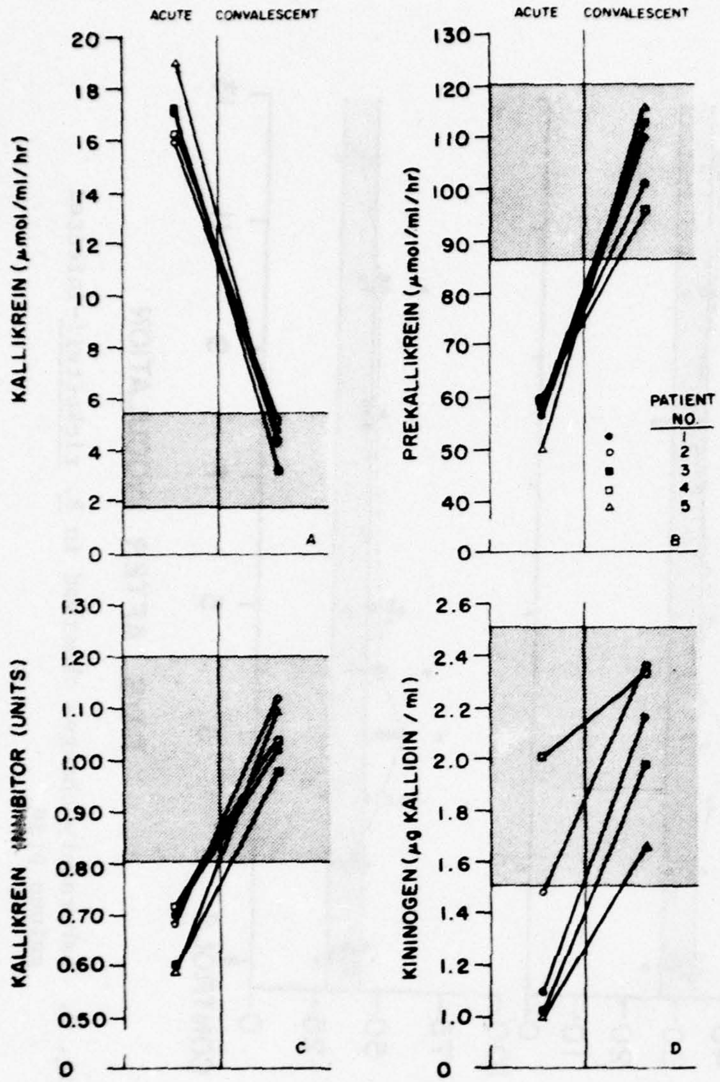

Fig. 3. Kinin system activation in 5 infected laboratory personnel

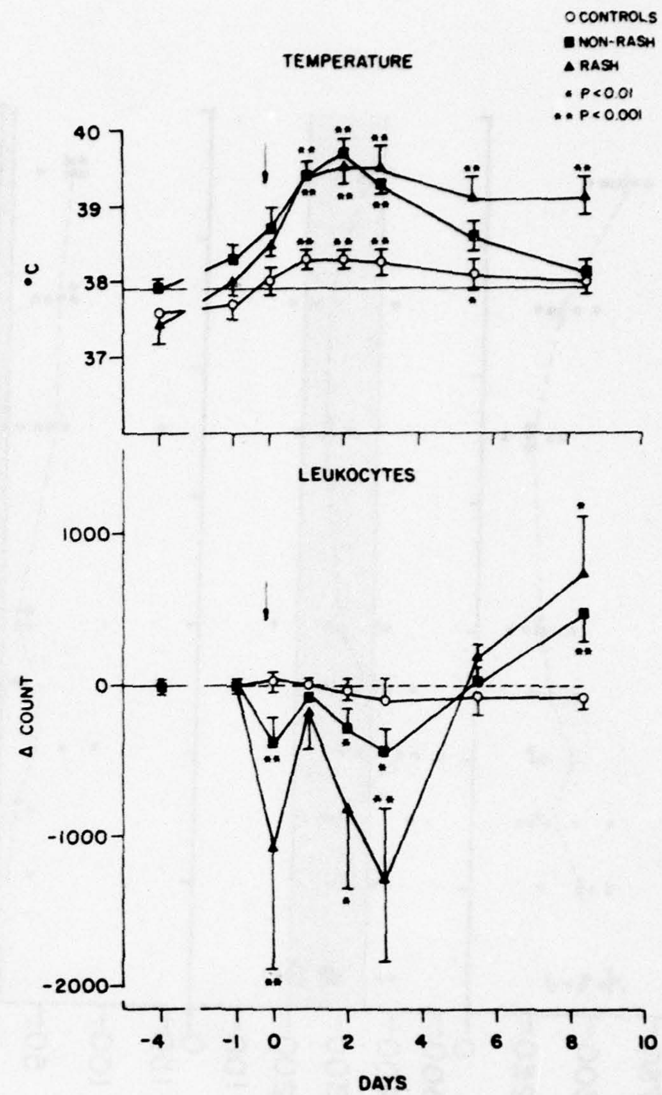

Fig. 4. Febrile and leukocyte responses in S. typhimurium septic monkeys with without rash. Arrow indicates time of infection

In general, the magnitude of coagulation changes could be correlated among the 3 groups of monkeys (noninfected control, infected without rash, and infected with rash) to the level of bacteremia. The group with rash had the greatest change. Activation of the coagulation system was suggested by the large decrease in platelet levels of the infected monkeys developing rash (Fig. 5).

Activation of the coagulation system was also suggested by prolongations of both PT and APTT at the height of illness (Fig. 6). Both groups of infected monkeys had prolonged coagulation times, while the control monkeys tended toward somewhat shorter times. This shortening was probably due to the stress of daily bleedings.

All infected monkeys had increased levels of fibrinogen throughout the infection (Fig. 7). This is to be expected, since fibrinogen is an acute-phase reactant protein. It is also consistent with what others have seen, that fibrinogen levels rarely fall in DIC associated with infections. FDP were seen in the serum of all the infected monkeys, but those which developed rash had much higher levels than those which did not. This suggests significant fibrinolytic activation in all infected monkeys.

Kinin system activation was suggested by significant drop in prekallikrein levels of infected monkeys (Fig. 8). Again, infected monkeys with rash had the greatest change. The small, although apparently significant, drop in PK levels of the control group of monkeys is within 2 SD of the preinfection base-line value.

In addition to this clinical and laboratory evidence of DIC, pathologic evidence of DIC was found in the one infected monkey which died at the height of illness. Multiple fibrin thrombi were found in the glomerular tufts of both kidneys, as well as in other tissues.

Having developed a useful, reproducible model for DIC associated with gram-negative sepsis, we went on to study the metabolic behavior of selected proteins during infection and DIC.

The normal disappearance of a labeled plasma protein is biphasic as shown in Fig. 9. The disappearance of the labeled protein can be described by the double exponential equation, one term representing net equilibration between intravascular and extravascular pools, and the other the net rate of metabolism.

Labeled homologous fibrinogen disappears from the plasma of noninfected monkeys with the kind of behavior described (Fig. 10). The mean half-life of the fibrinogen, which is proportional to the slope of the straight portion of the curve for this group is 44-48 hr. The disappearance of labeled fibrinogen from the plasma of infected monkeys can in a way also be described by a double exponential equation; however, the apparent half-life is shorter. For infected monkeys which did not develop rash, the half-life was 42 hr, for those developing rash, 30 hr.

Fractional catabolic rate is another measure of protein metabolism, which reflects the distribution of a protein as well as its half-life (Table I). For labeled fibrinogen, control monkeys utilize 56% of their intravascular

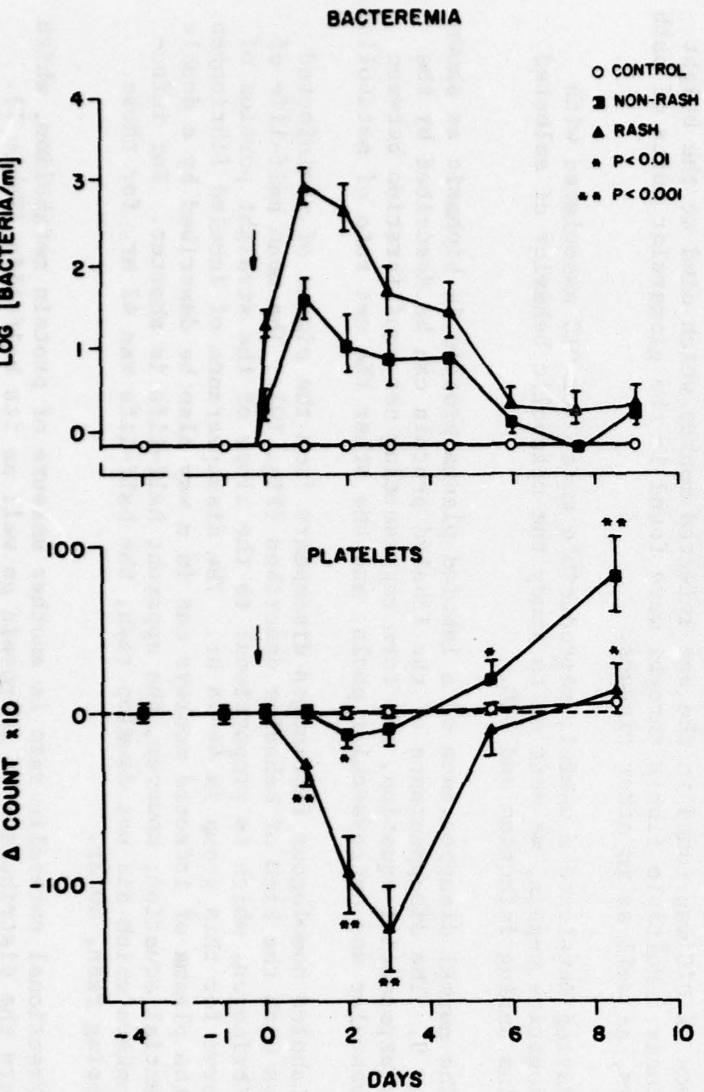

Fig. 5. Bacteremia and platelet response in _S. typhimurium_ septic monkeys with and without rash.

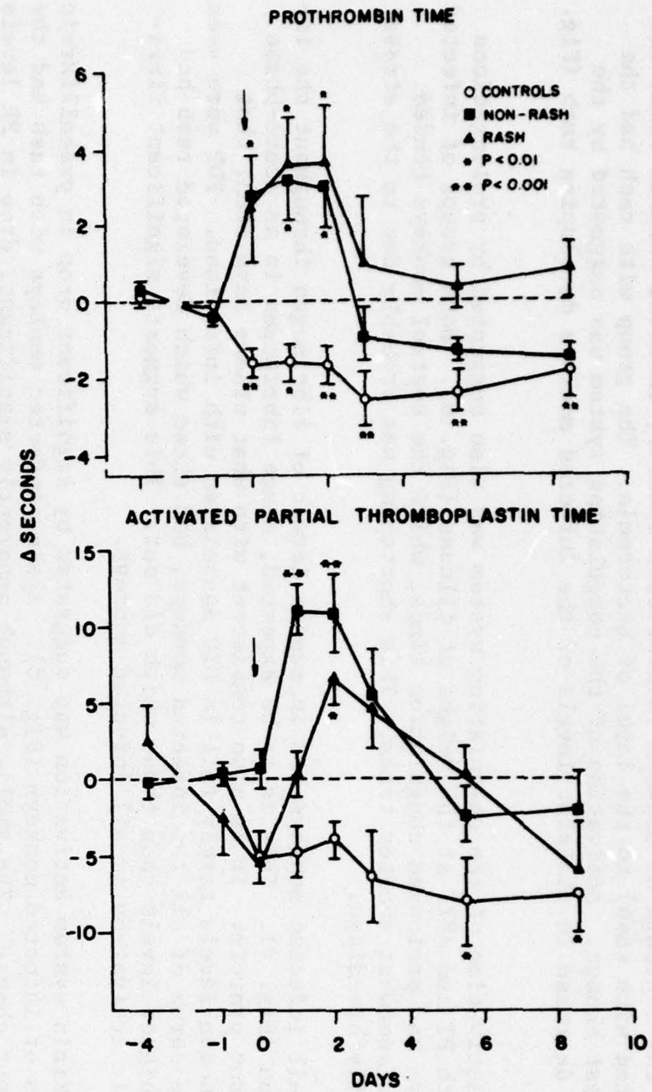

Fig. 6. Prothrombin and activated partial thromboplastin times in _S. typhimurium_ septic monkeys with and without rash

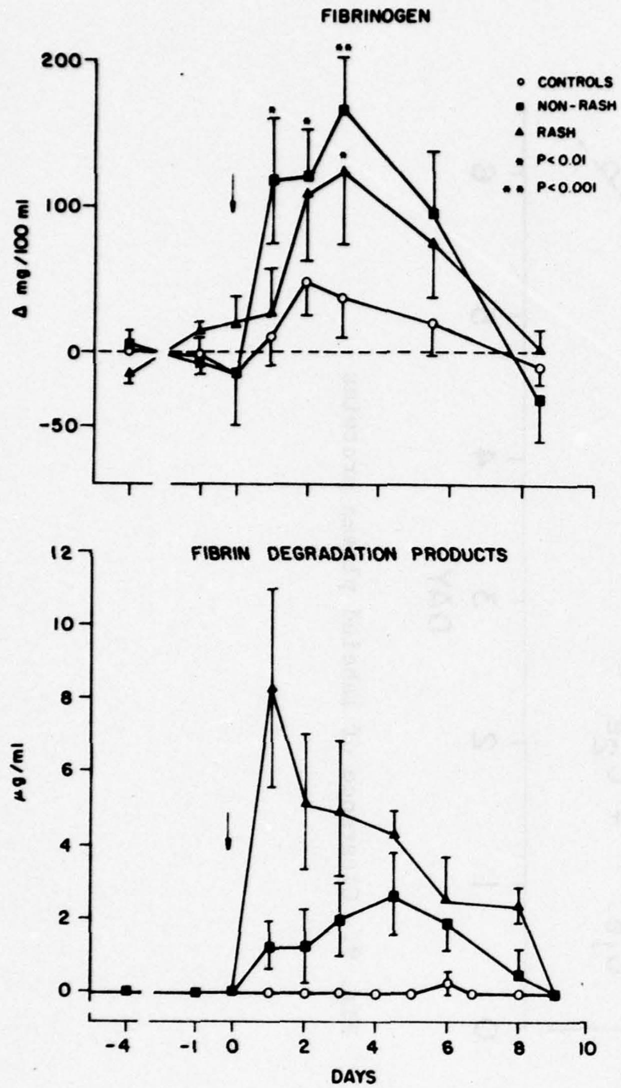

Fig. 7. Fibrinogen and fibrin degradation products in S. typhimurium septic monkeys with and without rash

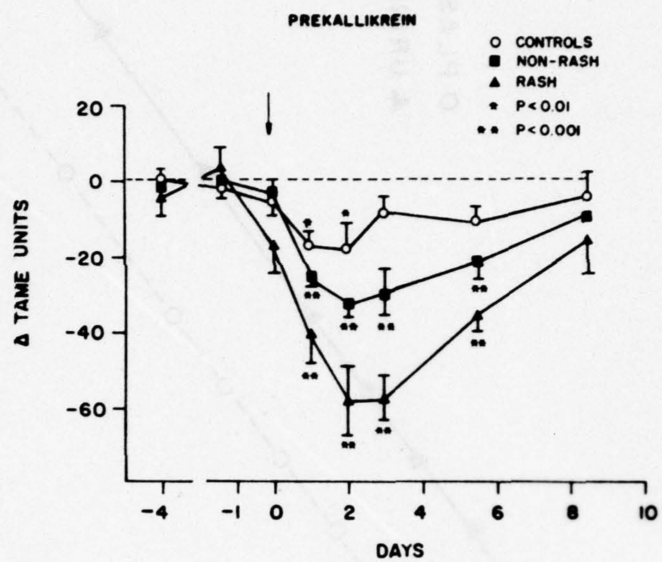

Fig. 8. Prekallikrein levels in S. typhimurium septic monkeys with and without rash

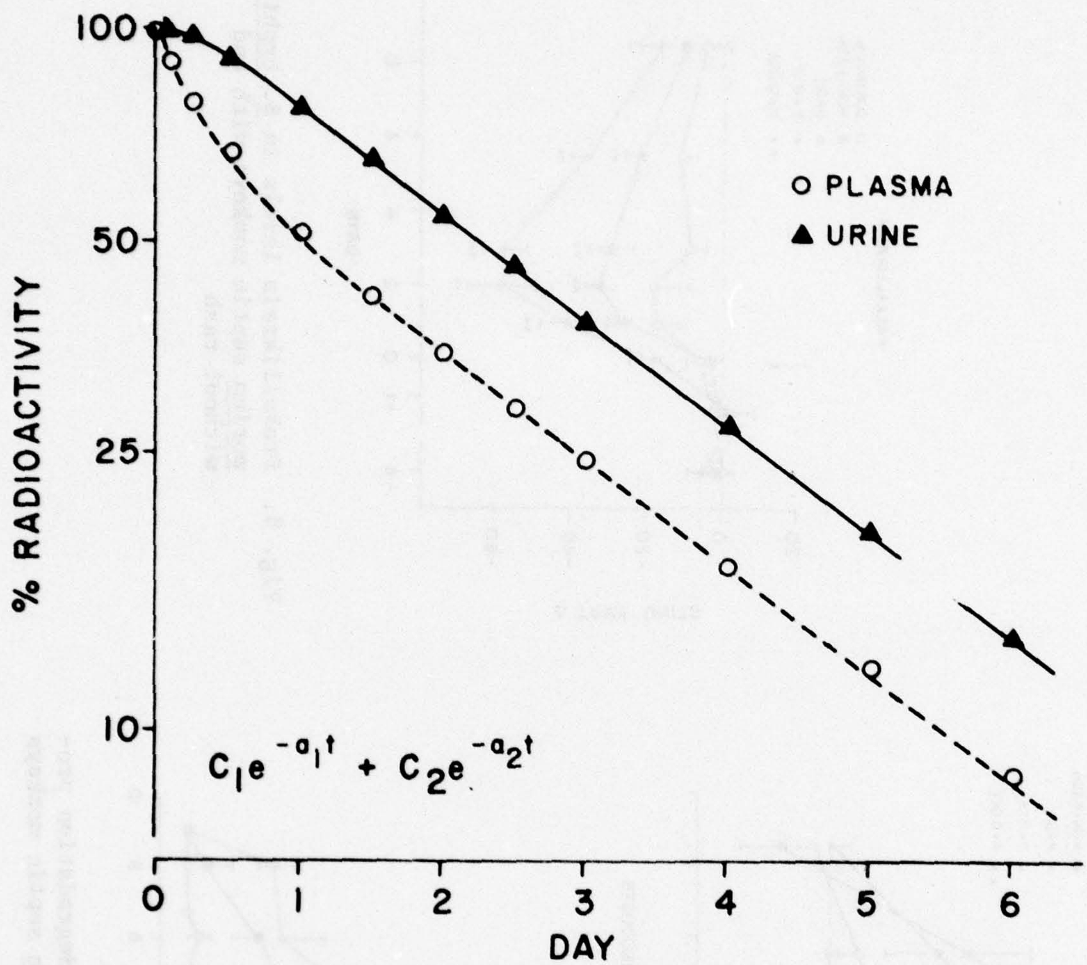

Fig. 9. Clearance of labeled plasma proteins

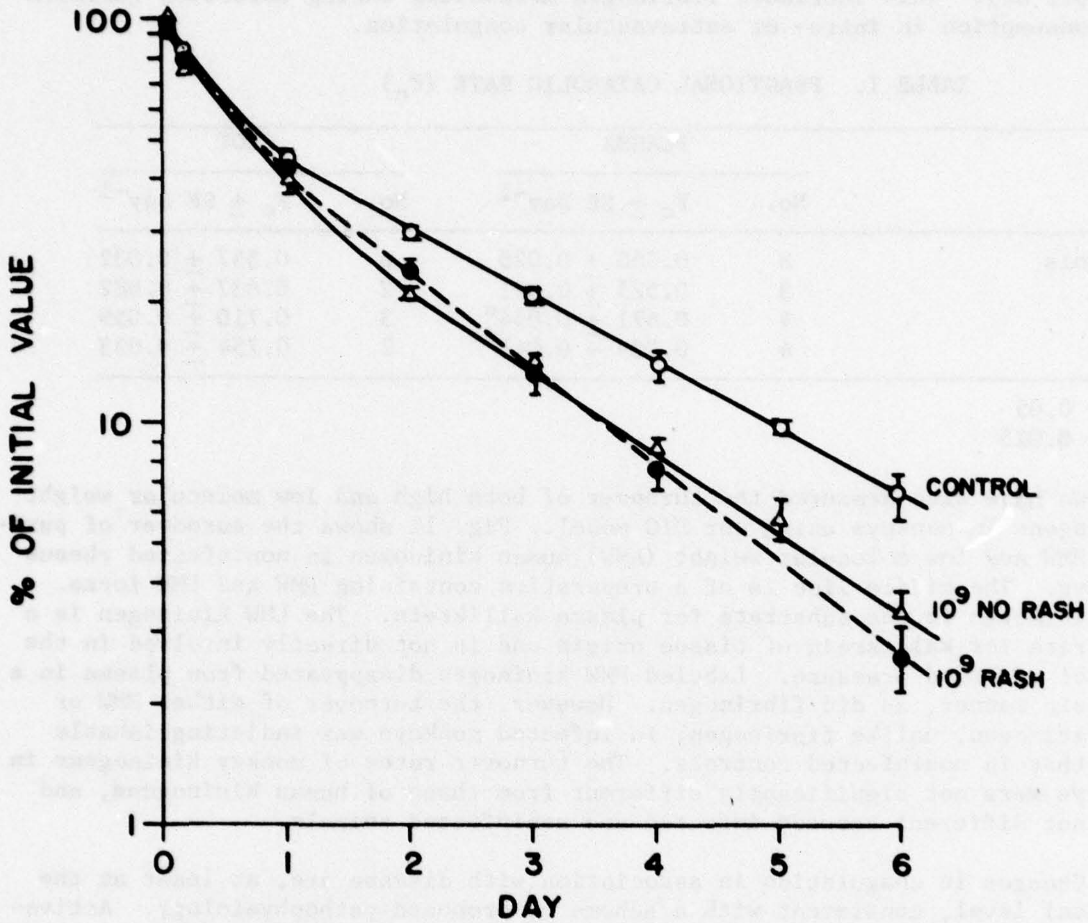

Fig. 10. Disappearance of labeled homologous fibrinogen from S. typhimurium-infected monkeys

fibrinogen per day. Infected monkeys without rash utilize more, 60-70%, and infected monkeys which develop rash utilize 75% of their intravascular fibrinogen per day. This increased fibrinogen metabolism during infection reflects its consumption in intra- or extravascular coagulation.

TABLE I. FRACTIONAL CATABOLIC RATE (F_c)

GROUP	PLASMA		CLOT	
	No.	$F_c \pm$ SE Day^{-1}	No.	$F_c \pm$ SE Day^{-1}
Controls	8	0.560 ± 0.025	6	0.557 ± 0.032
10^8	3	0.525 ± 0.051	2	0.637 ± 0.082
10^9	4	0.671 ± 0.054*	3	0.710 ± 0.059
Rash	4	0.729 ± 0.041**	2	0.754 ± 0.033

*P < 0.05
**P < 0.025

We have also measured the turnover of both high and low molecular weight kininogens in monkeys using our DIC model. Fig. 11 shows the turnover of purified HMW and low molecular weight (LMW) human kininogen in noninfected rhesus monkeys. The middle line is of a preparation containing HMW and LMW forms. HMW kininogen is the substrate for plasma kallikrein. The LMW kininogen is a substrate for kallikrein of tissue origin and is not directly involved in the control of blood pressure. Labeled HMW kininogen disappeared from plasma in a biphasic manner, as did fibrinogen. However, the turnover of either HMW or LMW kininogen, unlike fibrinogen, in infected monkeys was indistinguishable from that in noninfected controls. The turnover rates of monkey kininogens in monkeys were not significantly different from those of human kininogens, and were not different between infected and noninfected animals.

Changes in coagulation in association with disease are, at least at the clinical level, consistent with a scheme of proposed pathophysiology. Activation of the coagulation system is reflected by increased PT and PTT times and decreased platelet counts. Fibrinogen levels are less useful in infections, since turnover studies show increased fibrinogen metabolism despite elevated levels of fibrinogen in gram-negative sepsis associated DIC.

Activation of the fibrinolytic system is indicated by increased FDP in the serum. Several investigators have noted FDP accompanying severe infection, even if there were no clinical indications of DIC (i.e., rash).

Kinin system activation is suggested by lowered PK levels. Significant kinin system activation was suggested in even mild cases of RMSF acquired in the laboratory, where other coagulation system changes were minimal. This implies that kinins may play a possible role in the vasculitis seen in the early stage of this disease.

Further insights into the pathophysiology of this syndrome may come from our studies of the changes in metabolism of selected proteins during infection using our model of sepsis-associated DIC. We have noted an increased rate of

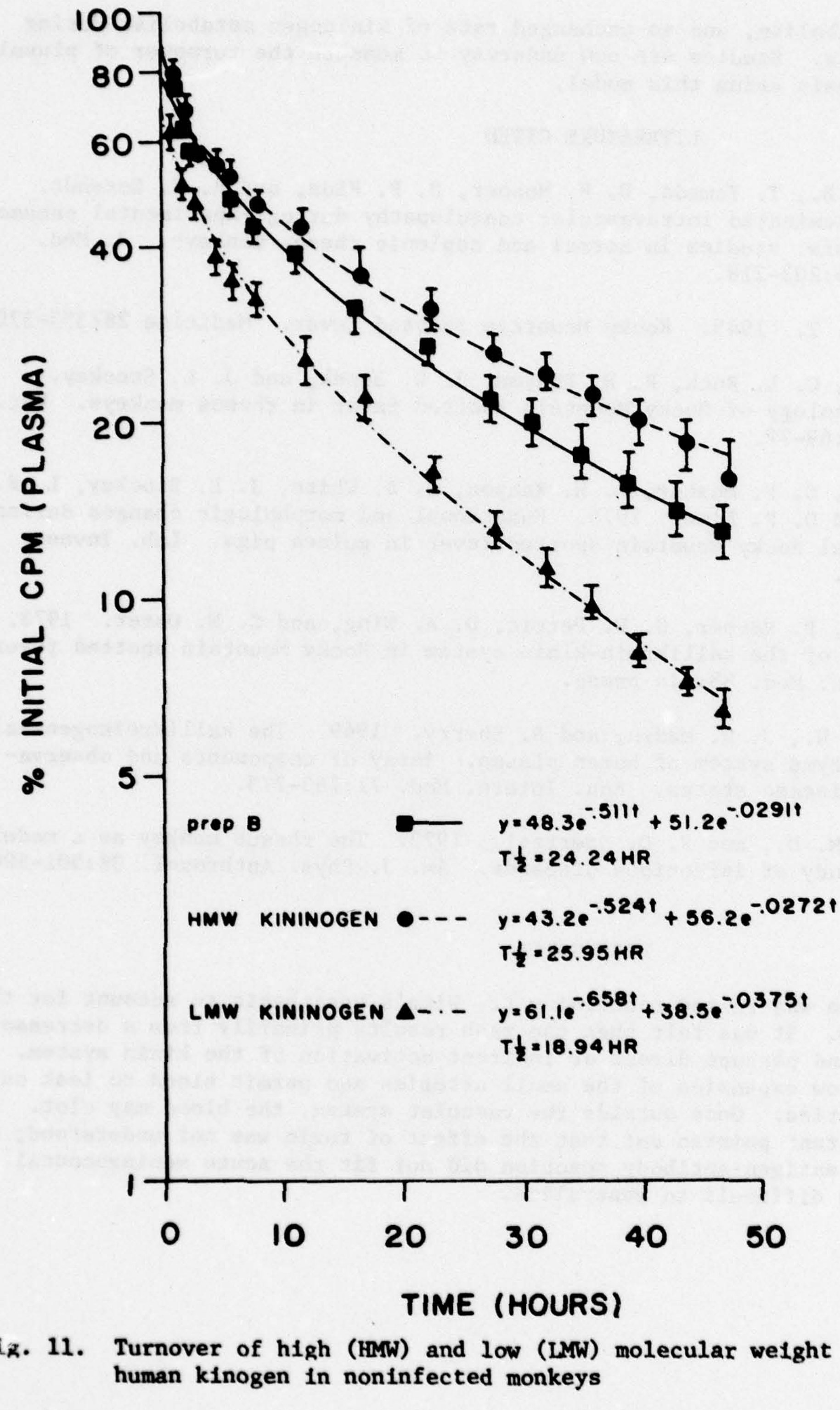

Fig. 11. Turnover of high (HMW) and low (LMW) molecular weight human kinogen in noninfected monkeys

fibrinogen metabolism, and an unchanged rate of kininogen metabolism during bacterial sepsis. Studies are now underway to measure the turnover of plasminogen during sepsis using this model.

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DISCUSSION

A question was raised concerning Dr. Wing's hypothesis to account for the petechial rash. It was felt that the rash results primarily from a decrease in platelets and perhaps direct or indirect activation of the kinin system. This would allow expansion of the small arteries and permit blood to leak out of the capillaries. Once outside the vascular system, the blood may clot. Another consultant pointed out that the effect of toxin was not understood; moreover, the antigen-antibody reaction did not fit the acute meningococcal model. It was difficult to generalize.

CELLULAR IMMUNE RESPONSES IN THE EVALUATION OF VACCINES

Michael S. Ascher, MC

Studies on the cellular immune response to infectious diseases have recently proliferated with the development of rapid, quantitative, in vitro test procedures for assessment of lymphocyte function in man and experimental animals. One basic assumption underlying these endeavors is that a cellular immune response is of some value in resistance to infection. There is, however, little direct experimental evidence correlating the state of cellular hypersensitivity of an individual and protective immunity. Studies that have been done have concentrated on ablating the cellular arm of the immune system with radiation or antithymocyte serum, for example, and measuring an animal's response to subsequent infection. There is little information on what effects measures designed to augment cellular immunity produce in infectious models. This is partly because it is very difficult to produce elevated levels of delayed-type hypersensitivity or cell-mediated immunity in experimental animals as the early history of transfer factor in animals illustrates. We have recently had the opportunity to investigate the effects of a novel means of elevating cell-mediated immunity on the immune response to model vaccines and to correlate such efforts with protective resistance.

The studies described were conducted in the laboratory of Professor John Turk of the Royal College of Surgeons of England while I served as a travelling fellow of the Royal Society of Medicine Foundation for 3 months last spring. The basic experimental design is illustrated in Table I. Guinea pigs were immunized with antigen in Freund incomplete adjuvant (FIA). They were skin-tested at intervals after immunization and cellular and humoral immune studies

TABLE I. EXPERIMENTAL DESIGN

Day 3	Day 0	Day 7	Day 14	
Cytoxan 250mg/kg IP	Immunize (foot pads in FIA)	Skin Test LT Ab	Skin Test LT Ab	Challenge

were performed. Professor Turk has previously reported that the cytotoxic drug cyclophosphamide (cytoxan), widely used in cancer chemotherapy, produced selective depletion of B cell areas of lymphoid tissue of guinea pigs and mice when the drug was administered as a single large injection. Such changes were associated in functional studies with diminished antibody response to an appropriately timed immunization, in this case after a 3-day interval. Paradoxically animals so treated manifested an increased degree of delayed-type hypersensitivity and cellular immune reactivity. Thus a state of selective immune imbalance could be produced. One current interpretation of these results suggests that a population of suppressor B cells is eliminated by cytoxan treatment which results in a release of cellular immunity from its physiologic constraints.

This effect of cyclophosphamide pretreatment has been reported in a large variety of antigen systems in mice and guinea pigs. The most frequently used antigens in previous studies have been purified proteins, such as ovalbumin or bovine gamma globulin. There are no reports of positive effects of cytoxan on reactivity to microbial vaccines. We therefore asked the questions: 1) can cytoxan potentiate delayed-type hypersensitivity to vaccine antigens? and 2) if so, what effect does such alteration of the immune response have on protective resistance? Fig. 1 shows the effects of cytoxan pretreatment on skin test responses to tularemia vaccine antigen. There are 2 subjective indices

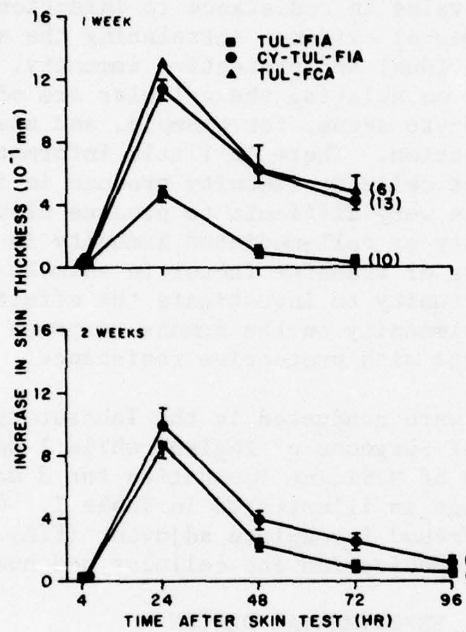

Fig. 1. Effect of cytoxan (CY) pretreatment of guinea pigs on skin test responses to tularemia vaccine (10 μg) as measured by skin thickness.

of delayed-type hypersensitivity reactivity commonly used, the diameter of the reaction and the intensity of the erythema. A more objective measurement is the specific increase in skin thickness at the skin test site. This measurement is made with a skin caliper and is calculated as the thickness of the skin test site minus the average thickness of normal skin on either side of the site. The results are expressed in tenths of a millimeter. The scale in the figures would also roughly indicate the diameter of reactions in millimeters. The upper and lower halves of this figure refer to measurements at 1 and 2 wk, respectively. In this experiment we compared the skin reactions produced in recipients of killed tularemia vaccine emulsified in FIA with and without cytoxan pretreatment. A positive control was included for reference, i.e., animals immunized with tularemia antigen in Freund complete adjuvant (FCA). As can be seen in the top half of the figure, when these 3 groups were skin tested 1 wk after immunization, cytoxan pretreatment resulted in a potentiated and prolonged skin reaction approaching that seen with vaccine administered in FCA. Such reactions were quite intense with a diameter of induration

of 15 - 20 mm, which occasionally proceeded to necrosis. In previous studies by Turk and others, the cytoxan effect on delayed-type hypersensitivity was short-lived and with ovalbumin, for example, B cell function rapidly returned resulting in Arthus reactions at 2 wk. In the tularemia system, however, no Arthus reaction was seen. Increased skin reactions persisted but was a function of the dose of antigen administered in the immunization. In this case the FIA group had increased at 2 wk and obscured the cytoxan effect. If, however, the immunizing dose was lowered 10-fold as shown in Fig. 2, significant immunopotentiation could be seen through week 3. Fig. 3 shows that the

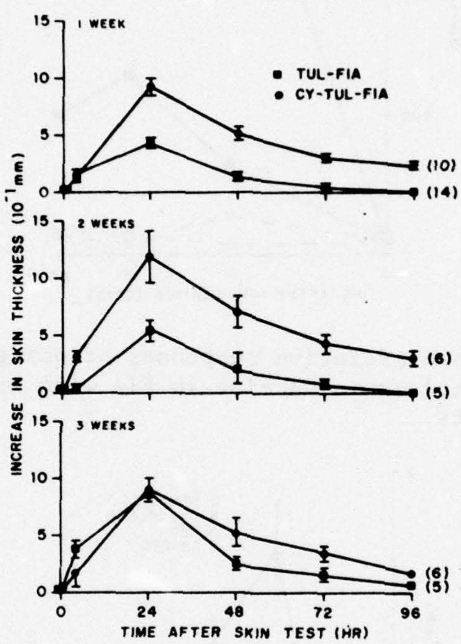

Fig. 2. Effect of cytoxan (CY) pretreatment of guinea pigs on skin test responses to tularemia vaccine (1 μg) as measured by skin thickness.

increase in skin reactivity was associated with an increased proliferative response of lymphocytes from animals pretreated with cytoxan. The background counts in the absence of antigen showed fluctuations due to the drug, but after the background had settled down, the increased response to antigen persisted. This *in vitro* result has also been seen in several other antigen systems. Fig. 4 shows that the elevated delayed skin reactivity was also associated with an increased ability to transfer such reactivity to normal unimmunized recipients along the lines of the classical Chase experiment which gave birth to cellular immunology. The experiment involves the IV injection of cells derived from immunized donors into normal recipients, followed by skin-testing of the recipients 1 hr later. We see that peritoneal exudate cells from cytoxan-pretreated donors were most active, followed by their spleen cells, and then by cells from untreated immunized donors.

Although the cytoxan treatment leading to heightened delayed skin reactivity in these experiments cannot be readily contemplated in man, it is worthwhile asking if an elevated cellular immune state, produced by whatever

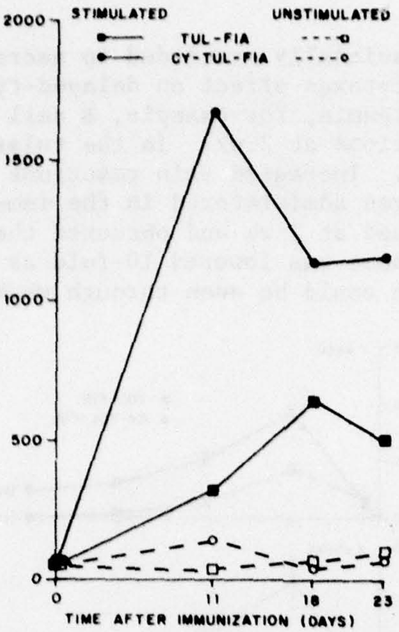

Fig. 3. Lymphocyte proliferative responses of guinea pigs immunized with 10 μg tularemia vaccine in FIA with or without cytoxan pretreatments.

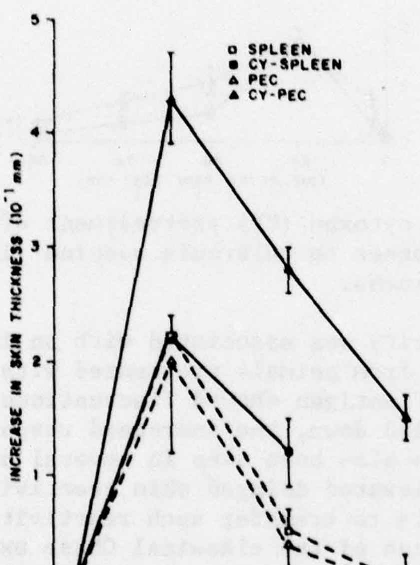

Fig. 4. Effect on skin reactivity of normal given peritoneal exudate cells (PEC) on spleen cells from donors immunized with or without cytoxan pretreatment.

mechanism, is associated with changes in protective immunity. This question is crucial to address before embarking on difficult trials of controversial cellular immune potentiators, such as transfer factor, levamisole or thymosin. As in the case of immune surveillance against cancer, even though it sounds like a reasonable idea, there is little direct evidence for it. Since returning to USAMRIID we have addressed the protective aspects of this model system. A tularemia challenge model cannot be readily used, since killed antigen in any form will not protect against the challenge strains employed most commonly.

Therefore we turned to a Q fever model in collaboration with MAJ Kishimoto, Aerobiology Division. Figure 5 shows the effects of cytoxan pretreatment on skin-test responses of guinea pigs immunized with the phase I Q fever vaccine prepared for human use. As in the case of tularemia, potentiation was seen to persist for a considerable period. We then immunized groups of animals without skin-testing and subjected them to an infectious challenge with a small-particle aerosol of Coxiella burnetii. As can be seen on Table II, there was no appreciable diminution of resistance due to cytoxan pretreatment after immunization. To summarize, immunization with vaccine in FIA lowered the median protective dose (PD_{50}) to one-third; cytoxan pretreatment lowered it another 4-fold.

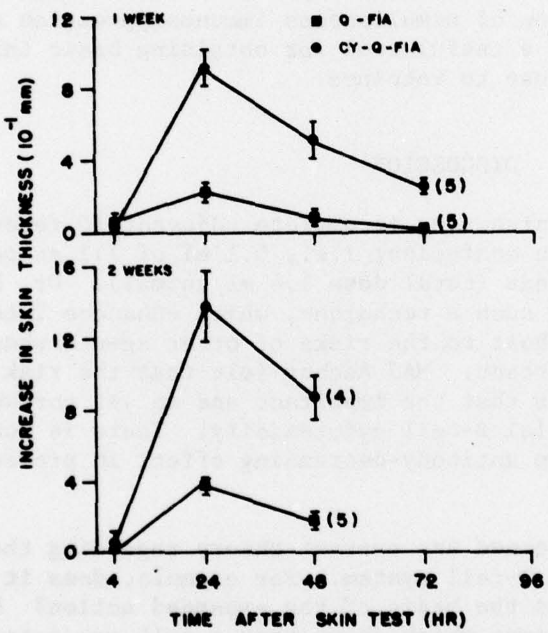

Fig. 5. Effect of cytoxan pretreatment of guinea pigs skin test responses to Q fever vaccine (Phase I) as measured by skin thickness

In other diseases where antibody plays a crucial role such as a model Pseudomonas infection, as reported by others, cytoxan pretreatment abolished the protective effects of immunization. The fact that protective resistance develops after cytoxan pretreatment is further evidence for the cellular immune nature of this resistance. If changes similar to those we have demonstrated in skin reactions or in vitro assays could be induced by adjuvants or

TABLE II. EFFECT OF CYTOXAN PRETREATMENT ON Q FEVER

PHASE I IMMUNIZING DOSE (μg/kg)	NO. FEBRILE/TOTAL (PD_{50}) None	Cytoxan 250 mg IP
0	5/5	6/6
0.03	3/3	4/5
0.3	4/5	2/5 (0.14)
3.0	0/5 (0.57)	0/5
Vaccine alone	(1.8)	

certain forms, dosages or immunization schedules of vaccines, we would by inference have reason to pursue the treatment as potentially useful. This model system with the simultaneous ability to measure cellular immunity and protective resistance would seem ideally suited for the comparative evaluation of adjuvants proposed for use in man. These studies will be extended to the numerous other viral, bacterial and rickettsial challenge models at USAMRIID in an attempt to determine the relative importance of cellular immunity in these diseases. The paradox of simultaneous immunosuppression and immunopotentiation by cytoxan will be a useful tool for obtaining basic information on the nature of the immune response to vaccines.

DISCUSSION

The technique of administering incomplete adjuvant (Q fever) was redescribed to eliminate some confusion; i.e., 0.1 ml of 1:1 suspension was injected into all 4 foot pads (total dose 1.4 ml/animal). Dr. Feigin raised the question as to whether such a technique, which enhances T-cell response, concomitantly exposes the host to the risks of other agents where the B-cell response is even more important. MAJ Ascher felt that the risk factor may, indeed, be raised. He felt that the important and as yet unresolved problem was why there is preferential B-cell cytotoxicity. There is some concern as to whether it really has an antibody-decreasing effect in preformed antibody situations.

Another question concerned the current theory regarding the mode of action of cyclophosphamide on the T-cell system. For example, does it hit a subpopulation of T-cells? What is the basis of the expanded action? MAJ Ascher replied that there is evidence which shows that a cell generated into "activity" in a normally immunized animal will then suppress the skin reaction of another normal animal. The cell which is suppressive bears immunoglobulin. If the animal is pretreated with cytoxan, that cell is not present. The reason for the "knockout" is not understood. Professor Turk wants to characterize this cell with a thymus antigen to determine if it is a postthymus cell with a peculiar immunoglobulin.

Another consultant asked if this cytoxan method was similar to the procedure developed 20 yr ago by Uhr and Pappenheimer whereby animals were made extremely hypersensitive by injecting small amounts of antigen-antibody

precipitates; the method did not kill 10% of the animals. MAJ Ascher replied that Turk believes this type of antigen-antibody complex reactivity is more like the Jones-Mote phenomenon, an interaction between 4-hr reactivity and delayed reaction. All cytoxan does in the model is to remove the antibody component. Turk is convinced that immunization with a complex has a component of the Jones-Mote reactivity present and this is not "pure" delayed sensitivity. For example, in the guinea pig, the JonesMote reaction is very active at 4 hr, peaks at 24, and goes away by 48 hr. Another consultant felt that this system was entirely different from the one employed by Uhr and Pappenheimer. He suggested the use of mice without T cells, since they have been used in studies designed to differentiate the roles of humoral and cellular immunity. He felt that this was important but difficult work.

A consultant described how typical tropical ulcers were formed in guinea pigs sensitized to diphtheria toxin when they were injected intradermally with virulent diphtheria bacilli. Delayed hypersensitivity walled off the infection and bacteria could be isolated at the bottom of the ulcer. These animals, when challenged, produced large amounts of antitoxin.

MECHANISMS OF ADJUVANT ACTIONS

Arthur O. Anderson, MC

The studies reported here were conducted in collaboration with Dr. Norman Anderson at the Johns Hopkins University School of Medicine, who is also a contractor supported by USAMRIID.

Recent developments in immunology clearly established that induction of immunity requires an intricate series of cellular interactions among classes of lymphocytes and monocytes. Recirculation of lymphocytes through lymph nodes (Fig. 1) may facilitate these interactions by providing constantly changing populations of immunocompetent T and B cells which pass through sites of antigen and macrophage concentration.

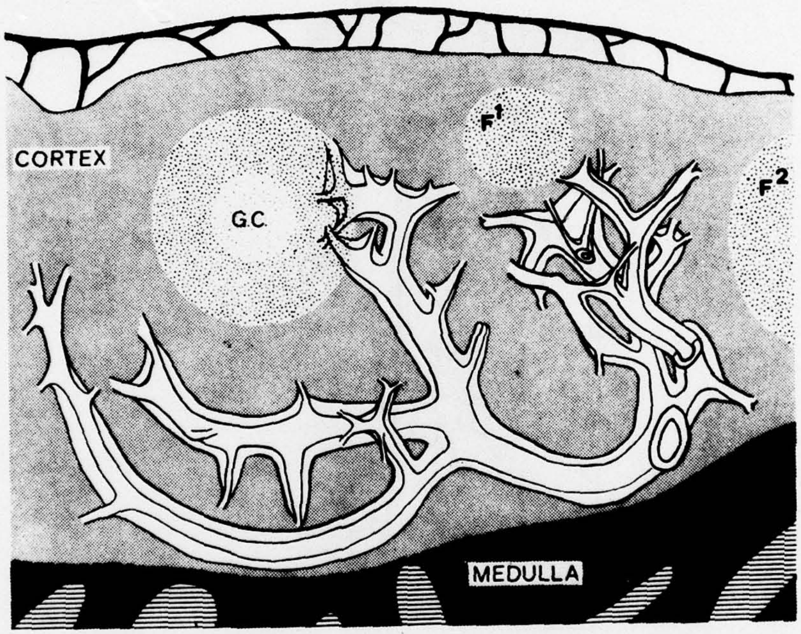

Fig. 1. Diagram of a lymph node. GC, germinal center; F^1, primary follicle; F^2, secondary follicle.

The magnitude of lymphocyte recirculation in a normal rat is sufficient to completely replace 1/3 of the lymphocytes in each lymph node every 24 hr. Study of the basic mechanisms governing lymphocyte recirculation through lymph nodes may provide clues to ways in which we may enhance immunity to microbial organisms.

Studies by Gowans and Knight (1) demonstrated that lymphocytes emigrate from the blood into lymph nodes by selectively crossing the walls of high endothelial venules (HEV). The present studies describe the basic anatomy, physiology and immunology of lymphocyte recirculation in normal and antigenically stimulated lymph nodes.

Since alteration in blood flow may influence lymphocyte recirculation, I will begin by briefly describing our studies of the lymph node microvasculature. Cleared sections from dye-perfused nodes sharply delineate the angioarchitecture and reveal specialized vascular structures. Arteriovenous communications in the cortical capillary arcade drained directly into high endothelial venules. HEV could be readily identified in these preparations because of characteristic staining of luminal and abluminal surfaces. Near the lymph node hilus, venous sphincters are found at junctions between lobular veins. Serial sections show that these sphincters are composed of circumferential bundles of smooth muscle.

Fig. 2 diagrammatically shows the relationship of arteriovenous communications, HEV and venous sphincters in the lymph node vasculature. The location of HEV between arteriovenous communications and venous sphincters may provide hemodynamic regulation of the distribution, accumulation and emigration of

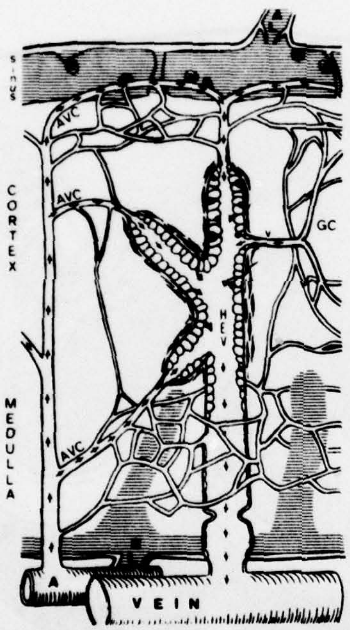

Fig. 2. Schematic of the relationship of arteriovenous communications, HEV and venous sphincters in a lymph node

lymphocytes in these venules. Fig. 3 shows the structure of an HEV wall. Studies of flow using labeled microspheres will be reported later.

The ratio of lymphocytes to red blood cells in HEV lumens is nearly 1000 times that of the peripheral blood, which suggests that lymphocytes are concentrated at this site. Others have incorrectly interpreted this to mean that lymphocytes entered the blood through HEV. Scanning electron microscopy (EM) showed that these lymphocytes were intimately attached to endothelial membranes by villous contacts. Perfusion studies suggested that these contacts were quite strong since hydrodynamic shearing forces sufficient to dilate venular lumens and flush away other blood elements *did not dislodge* adherent lymphocytes. Further, this membrane adhesion was maintained after immersion

in hyperosmolar solutions. Lymphocyte-endothelial cell contacts resisted osmotic stresses, which distorted cell contours.

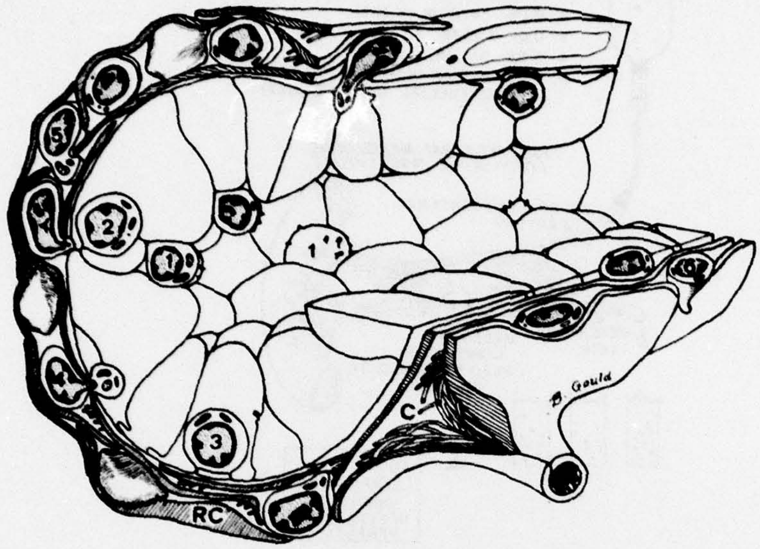

Fig. 3. Structure of an HEV wall.

The chemical nature of lymphocyte-endothelial cell adhesion is still uncertain. However, in EM preparations where special buffers were used to preserve surface oligosaccharides, fibrillar electron-dense material was seen traversing the 15-nm gap at these contact points. Regional perfusion techniques were used to obtain additional information about lymphocyte-endothelial cell interactions. Methods are shown in Fig. 4. Isolated rat mesenteric nodes were perfused intraarterially with dextran-saline solution until the venous effluent was clear. Then perfusion was switched to the fluids designated in Table I and maintained under controlled conditions for 12 min. The total number of effluent cells from these nodes was counted. Perfusate containing crystalline trypsin markedly increased the cell output when compared to control perfusion with dextran-saline. A similar increase in effluent cells was noted following ethylenediamine tetraacetic acid (EDTA) and normal saline perfusion. Histological examination of these nodes revealed that dextran perfusion failed to dislodge adherent lymphocytes, and left the endothelium and glycocalyx of HEV intact. The same perfusate containing 0.1% trypsin released all adherent cells without altering surface coat staining by alcian blue or producing endothelial cell damage. Perfusion with normal saline also dislodged adherent lymphocytes. This effect was probably related to the loss of a soluble component of endothelial glycocalyx which could be precipitated from the effluent perfusate. These results indicate that endothelial cell glycocalyx and lymphocyte glycoproteins interact via divalent cations to form the adhesive contacts shown in the EM studies. This correlates with the demonstration by Gesner and Ginsburg (3), Woodruff and Gesner (4, 5) that trypsin-treated lymphocytes do not recirculate through lymph nodes.

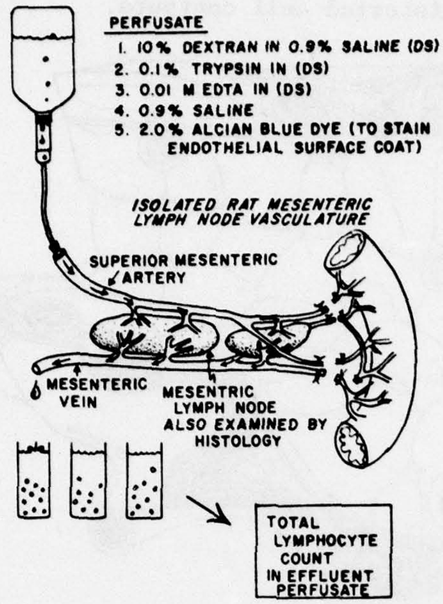

Fig. 4. Regional perfusion technique for studying lymphocyte attachment.

TABLE I. LOCATION OF RECIRCULATING LYMPHOCYTES DURING MIGRATION ACROSS HIGH ENDOTHELIAL VENULAR ENDOTHELIUM

METHOD	% EXTRACELLULAR (between cells)	NO. COUNTED
Standard EM*	80	467
Intraarterial Thorotrast	99	128
Intraarterial peroxidase	100	187
Intraarterial lanthanum	100	205

*Reference (2)

The next studies were done to clarify whether lymphocytes actively or passively crossed HEV endothelium, since Marchesi and Gowans (6) had proposed that these cells were engulfed and carried across inside endothelial cells. We approached this question by poisoning the lymphocyte's motile apparatus and examining the ability of these cells to accumulate in lymph nodes.

Normal tritiated uridine-labeled lymphocytes (U^3H Ly) rapidly crossed HEV walls and entered lymphatic tissue after IV infusion (Fig. 5). Pretreatment with the long-acting fungal toxin cytochalasin-A caused cytoskeletal effects which prevented movement across the endothelium, but did not prevent attachment of the lymphocytes to endothelial surfaces.

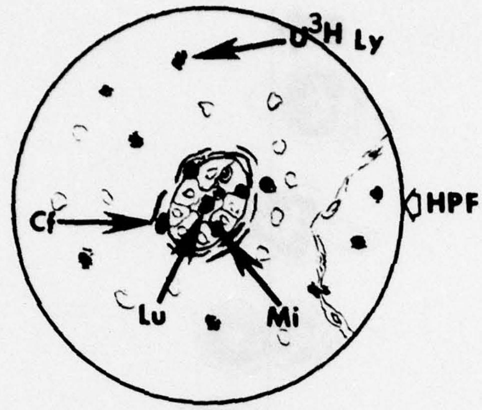

Fig. 5. Drawing of autoradiograph of migrating lymphocytes. Lu, lumen; Cf, cells in HEV cuff; Mi, migrating (intramural)

Quantitative data confirmed the inability of cytochalasin-A (cyto-A) to migrate. Untreated lymphocytes accumulated in significant numbers during the 3-hr interval after IV infusion, while trivial accumulation was seen after infusion of cyto-A treated cells (Fig. 6). The effect of cyto-A began to wear off after 4 hr, providing the experiment with a built-in viability control. Lymph nodes removed 24 hr postinfusion showed equal accumulations of normal and cyto-A treated cells. Apparently cyto-A prevented migration by producing cytoplasmic rigor and aggregation of actin-like filaments under the lymphocyte cell membrane. Inability of the treated lymphocyte to move its surface receptors in an orderly fashion caused blebbing and zeiosis along with restricted motility.

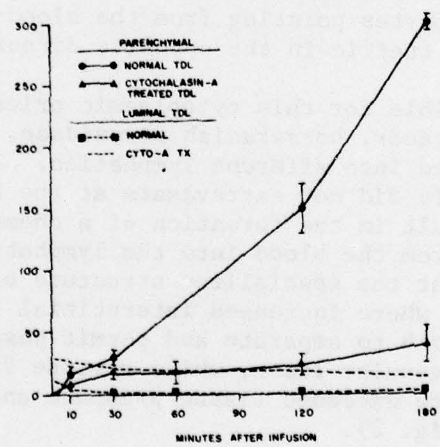

Fig. 6. Effect of cytochalasin-A treatment on lymphocyte (TDL) recirculation Tx = treated.

A few investigators have emphasized that autoradiographic studies do not preclude the possibility that some of the lymphocytes may be moving in the opposite direction. To resolve this query we attempted to assess the direction of migration morphometrically. Many years ago Lewis (7) described the motion of lymphocytes <u>in vitro</u> (Fig. 7). Lymphocytes moved forward with the nucleus

Fig. 7. Sequential structural changes in a lymphocyte during forward movement *in vitro* (after Lewis, Ref. 7)

leading and an organelle-rich uropod followed. Our ultrastructural studies used this observation to show that most migrating lymphocytes were fixed in a motile configuration which was oriented in the direction of migration (Fig. 8). This scattergram drawn on a 360° compass rose demonstrates the distribution of cytoplasmic polar angles and directional orientation of 203 migrating lymphocytes from electron micrographs of HEV. This distribution shows predominant orientation of lymphocytes pointing from the blood to the node parenchyma, which makes cellular traffic in the opposite direction highly unlikely.

What is responsible for this cytoplasmic orientation? We have found that the macromolecular tracer, horseradish peroxidase, rapidly entered HEV lumens after it was deposited into afferent lymphatics. In contrast, peroxidase injected intraarterially did not extravasate at the HEV. This unidirectional permeability may result in the formation of a chemotactic gradient which attracts lymphocytes from the blood into the lymphatic tissue. Ultrastructural studies indicated that the specialized structure of the HEV wall provided valve-like functions where increased interstitial fluid pressure could cause the perivascular sheath to separate and permit passage of fluid from the node parenchyma into the venular lumen, while reverse flow might be prevented when intravascular pressure exceeded tissue pressure and closed gaps between the overlapping cells (Fig. 2).

It is through these kinds of interdisciplinary studies that we have grown to understand how the normal rat lymph node works to regulate lymphocyte recirculation. The unusual microvasculature of the lymph node provides hemodynamic regulation of lymphocyte recirculation by affecting nodal blood flow, tissue and vascular hydrostatic pressure, and permeability of HEV. This produces optimal conditions for selective adhesion of lymphocytes on HEV surfaces and chemotaxis of these immunocompetent cells into lymph nodes.

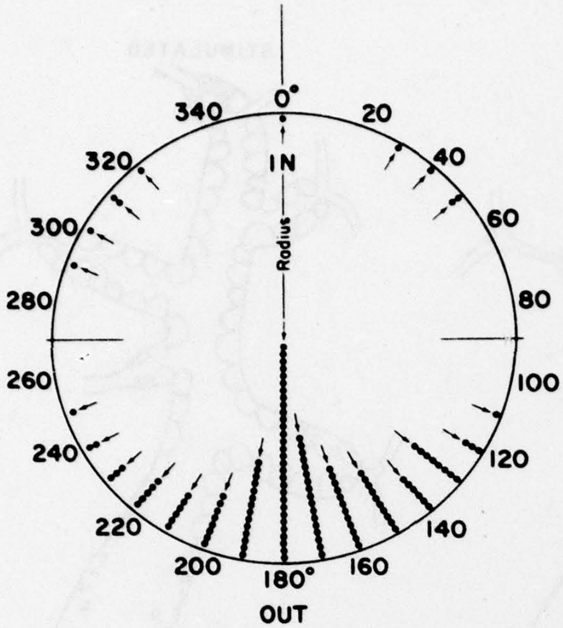

Fig. 8. Direction of migration by cytoplasmic polarity

In an antigen-stimulated lymph node, are these phenomena merely increased or is the story more complicated? In antigen-stimulated lymph nodes we have observed elongation and increased arborization of high endothelial venules, which appears to increase the surface available for lymphocyte emigration (Fig. 9). This observation of angiogenesis was confirmed using continous labeling with [^{3}H]thymidine. Autoradiographs revealed 25% labeling of HEV endothelial cell nuclei in antigen-draining nodes, while only 6% of HEV endothelial cells were labeled in contralateral controls.

Now that we have introduced all factors that might influence lymphocyte recirculation, let us examine what actually happens to lymphocyte traffic during strong antigenic stimulation.

Fig. 10 illustrates diagrammatically the influx, efflux, and retained populations of lymphocytes during the 54-day course of an immune response to derived sheep red blood cells, and correlates this traffic with nodal blood flow and HEV proliferation. The lymphocyte traffic into the node shows a bi-modal pattern which peaks on days 3 and 14 postinoculation. The first peak appears to represent increased entry and exit of migrating cells. A small blip of exiting specific plaque-forming cells can be seen. These cells are probably going to be sequestered in the spleen and other peripheral lymphatic tissues to serve as inducer-cells for the second "specific" increase in traffic. The second peak occurs in the regional nodes draining antigen and in the total body lymphoid tissue mass. This peak is associated with the accumulation within these nodes of specific antibody-forming cells. The first traffic peak is preceded by rapid changes in regional node blood flow which are probably due to release of inflammatory mediators or lymphokines within the node. A transient shut-down of exiting cells in the regional node directly correlates with these flow changes. This shut-down phenomenon has been termed

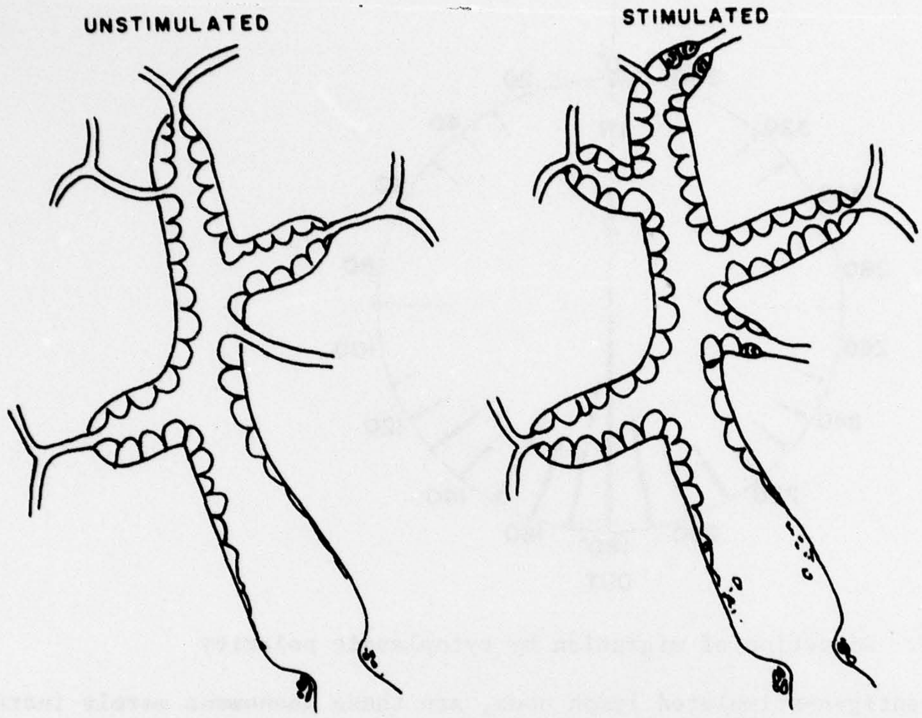

Fig. 9. Increased arborization of HEV stimulated with an antigen

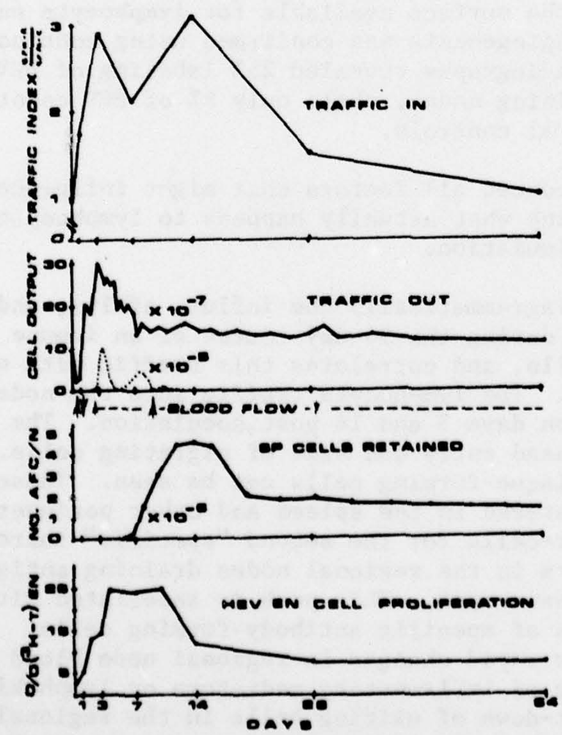

Fig. 10. Diagram of effect of antigen on lymph nodes

trapping, or nonspecific recruitment. The appearance of exiting specific plaque-forming cells appears to correlate in time with HEV endothelial cell proliferation; certainly HEV expansion precedes increased entry of specific antibody forming cells. This second phase of specific lymphoid traffic has been termed specific recruitment.

It is possible that HEV endothelial cell proliferation is necessary for an effective natural immune response; obviously further studies are necessary. This presentation has attempted to deal only with the less understood phenomena of lymphocyte traffic, but acknowledges the well-known phenomena of clonal selection, clonal expansion and maturation which have been studied so thoroughly using in vitro techniques. Perhaps the in vivo site of cellular cooperation between B cells, T cells and macrophages is in the wall of the HEV.

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DISCUSSION

A consultant asked whether, in the graft model used, the regional node effect was not related to the entrance of passenger lymphocytes from the graft into the regional node. MAJ Anderson replied that such entry was possible. However, most of these lymphocytes undergo degeneration within the graft before lymphocyte "hookups" occur. It is more likely that lymphocyte-associated histocompatibility antigens are carried to the node by the host macrophages, which invade the graft bed early in the response. Another consultant asked if the nodal HEV have relevance to species other than the rat? MAJ Anderson replied that they were first described in 1889 by the German anatomist Thome in the monkey. Subsequently, MAJ Anderson has observed HEV in human lymph nodes, and granuloma walls of rabbits, rats, guinea pigs, hamsters and mice. Bede Morris in Canberra, Australia, said that sheep and other cloven-hoofed animals do not appear to have HEV. They also have other differences in

the structure of their lymphatic tissues which make them heterologous to primates. The question was raised as to whether the presenter meant to indicate that sheep were not good models to use for analogy of immunity in man. MAJ Anderson did mean to indicate this with respect to the structure of the lymphatic system and the mode of lymphocyte recirculation. Antigen recognition, presentation and maturation of antibody-forming or cytotoxic cells may be analogous, but how these cells come together for cooperative interactions, and where they enter and leave the blood or lymphatic circulation may be different.